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EFFECT OF THYROXINE ON METABOLISM OF GLUCOSE AND AMINO ACIDS BY RAT KIDNEY CORTEX[1]

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ABSTRACT

Kidney slices from thyroidectomized rats incubated in Ringer-amino acid media synthesized considerable amounts of glucose, especially from L-proline. With added thyroxine the amount of glucose found slightly exceeded that of the controls when incubated at 37°. When slices were incubated at 5 and 37° the amount of glucose found in the thyroxine vessels greatly exceeded that of the controls. Similar differences existed in the oxygen consumption values. Thyroxine appeared to protect enzyme systems involved in oxygen consumption as well as gluconeogenesis from deterioration at 5°. Thyroxine effects, although noted at 37°, were always associated with an otherwise metabolically failing system.

THE addition of certain amino acids to Krebs Ringer solution produced a marked increase in oxygen consumption of kidney cortex slices from thyroidectomized rats (1, 2). With various substrates, the metabolic rate was better maintained in the presence of thyroxine (T4) than in its absence (1, 3). Because this T4 effect was greatest in the presence of proline (1), studies are here reported of respiratory and carbohydrate changes during prolonged periods of incubation.

Kidney tissue is reported to increase synthesis of carbohydrate in the presence of such compounds as pyruvate, acetate, lactate, butyrate, fumarate, malate, succinate, α-ketoglutarate, acetoacetate, glutamate, alanine, acetone bodies, and glycerol (4–9). Unpublished results from this laboratory permit the addition of sorbitol, ornithine and proline.

Although glucose has been widely used as substrate for a variety of tissues, we have found its usefulness very limited in maintaining metabolism of kidney cortex. It has little effect upon oxygen consumption and we

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concur with the findings that only slight utilization can be demonstrated 4, 5, 10). There is also evidence that net disappearance can only be demonstrated after depletion of endogenous glucose precursors or at a time when the tissue was otherwise metabolically failing. This failing system is further necessary for demonstration of a clear-cut *in vitro* T4 effect. An effect wherein maintenance of respiration appears more intimately connected with processes related to gluconeogenesis than glucose oxidation.

METHODS

Thyroidectomized male Sprague-Dawley rats were used following postoperative periods of at least six weeks. While under light ether anesthesia, they were sacrificed by exsanguination from the abdominal aorta. Both kidneys were quickly removed, placed in cold Krebs Ringer solution and cortex slices prepared according to the Deutsch technique (11). The 55 to 57 mg. fresh weight of tissue was added to each chilled Warburg vessel containing 2.7 ml. of Krebs Ringer incubation medium with glucose (580 to 609 μg.) or an amino acid (0.02 M). As described previously (2), T4[2] was added as 0.3 ml. of 1.3×10^{-2} mM in 0.005N NaOH and 0.9% NaCl. A corresponding control (blank, Bl) solution was used for the non-T4 vessels. Care was taken that the initial pH of all complete mixtures of solutions was 7.50 ± 0.05. When additional proline was to be tipped in, 0.165 ml. of 0.4 M solution adjusted to pH 7.50 was placed in the sidesacs of appropriate vessels.

When their contents were complete, the vessels were flushed with oxygen and mounted on the manometers. After a 15-minute 37° equilibration at a shaking rate of 90 per minute, oxygen consumption was measured over one hour. All Q_{O_2} values are μl. of oxygen consumed/mg. wet weight tissue/hour. The vessels to be kept in the cold between Q_{O_2} determinations were immediately placed in an ice and water bath upon withdrawal from the 37° temperature. They were then flushed with oxygen, capped with Parafilm and placed in a cold room at 5°. For any succeeding determination, the vessels were again flushed with oxygen and equilibrated for 15 minutes at 37° before starting the oxygen consumption measurement over at least one hour. Insignificant bacterial contamination was found under these circumstances, but special precautions were necessary in experiments involving 37° incubation for longer than six hours. Sodium penicillin G and streptomycin sulfate were added in amounts of 184 μg (300 units) and 300 μg per vessel, respectively. These amounts did not alter the oxygen consumption or other metabolic activity of tissue slices incubated up to 6 hours at 37° or for 3 days at alternating temperatures of 37° and 5°, as described above.

For analyses, the Warburg vessels were immediately placed in an ice and water bath, the tissue removed and the vessel contents centrifuged to remove any particulate matter remaining in suspension. In some instances, the tissue was homogenized in the incubation medium and the supernatant used. Protein was precipitated with trichloroacetic acid. Carbohydrate was determined according to the anthrone method of Hanson *et al.* (12). Comparison with a glucose oxidase procedure (13) demonstrated the carbohydrate in several representative solutions to be glucose. Recoveries of C^{14}-glucose by paper chromatography after incubation experiments using universally labeled C^{14}-proline were in agreement with the chemical determinations (unpublished results). The "initial carbohydrate" level was that present in a homogenized tissue aliquot not loaded into a Warburg vessel.

[2] The L-thyroxine used in this study was a gift from Dr. H. L. Fevold, Baxter Laboratories, Morton Grove, Illinois.

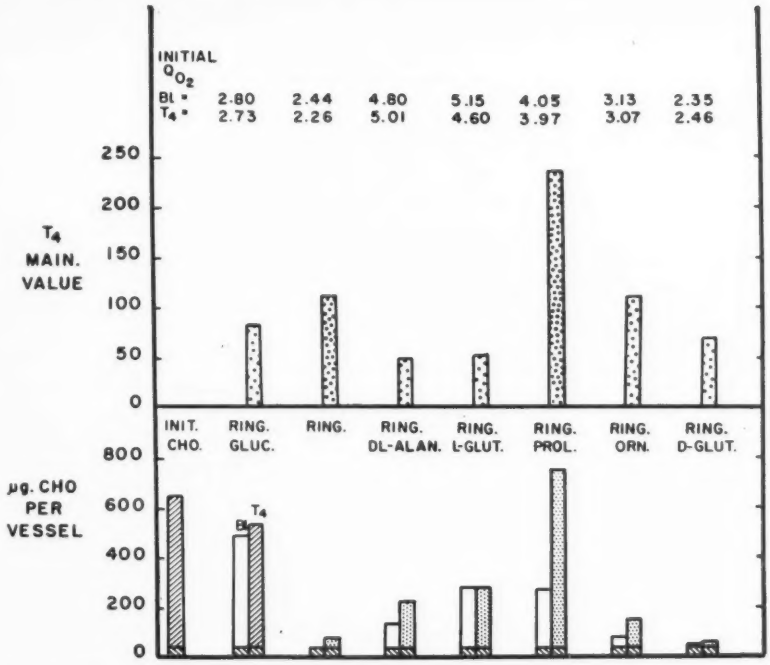

FIG. 1. Effect of various substrates on metabolism and gluconeogenesis of rat kidney cortex incubated for 3 days at 5° with appropriate periods each day at 37° for measurement of oxygen consumption. Above is shown each initial Q_{O_2} for a substrate yielding the stippled bar value for its Thyroxine (T4) Maintenance Value, a quantitative expression of T4 superiority over controls (B1). Below, paired bars represent the amount of glucose present after incubation in each medium containing blank (B1) or thyroxine (T4). The 45 μg starting endogenous carbohydrate level is shown by the small cross-hatched portion of each bar. The single bar at the lower left represents the starting carbohydrate level in the instance when 600 μg of glucose were added to the 45 μg of endogenous carbohydrate.

RESULTS

Incubation at Alternating Temperatures of 37° and 5° C

Support of respiration and gluconeogenesis by different substrates (Fig. 1). The initial Q_{O_2} was lowest with Ringer, Ringer-glucose and Ringer-D-glutamic acid as shown at the top of Figure 1. In contrast, the addition of L-ornithine, DL-alanine, L-proline or L-glutamate increased the initial Q_{O_2}. In no instance was there a significant difference between the blank (B1) and thyroxine (T4) vessels at this time.

The upper bar graph plots the "T4 Maintenance Value" (2). This is the sum of the differences between B1 and T4 determined after 1, 2 and 3 days

of incubation, based on the initial Q_{O_2} in each substrate shown. As the name suggests, the Maintenance Value is a quantitative expression of T4 superiority over each appropriate control. Although all these substrates supported an effect of T4 on respiration, only proline was better than Ringer (endogenous) or Ringer-ornithine in maintaining the initial Q_{O_2}.

The far left bar below ("Init. CHO") shows the total starting carbohydrate in the vessels containing 600 μg of glucose added at the start plus the initial determined tissue value (45 μg). The remaining lower columns represent the quantities of glucose present at the end of each 3-day experiment, comparing blank vessels with T4 for each medium shown. The first pair of bars ("Ring. gluc.") demonstrates disappearance of glucose from the Ringer-glucose solution. The slightly larger quantity remaining in the T4 vessel over that in the blanks is duplicated in the Ringer (no added glucose), suggesting greater synthesis from endogenous precursors rather than depressed oxidation. No net carbohydrate synthesis was obtained from D-glutamate and only small amounts from ornithine. DL-Alanine yielded somewhat more glucose, as did L-glutamate, B1 and T4, and L-proline without T4. Proline proved greatly superior to all other substrates in its ability to support gluconeogenesis in the presence of T4, corresponding to its much higher T4 Maintenance Value. The reason for choosing 600 μg of glucose in the Ringer-glucose experiments was to duplicate the carbohydrate levels reached during several preliminary incubations in Ringer-proline. It will be noted that the average glucose value in the Ringer-proline vessels shown in Figure 1 was actually 770 μg.

Thyroxine protection against deterioration following cold exposure. In Figure 2, all vessels were incubated in L-proline at 37° for 1¼ hours at the start. The values for Day 0 exhibited no differences between blank and T4 vessels, either in Q_{O_2} or carbohydrate levels. However, after an intervening 23 hours at 5°, an additional incubation for 1¼ hours at 37° produced a 45% fall in Q_{O_2} of the B1 vessels whereas the T4 vessels increased 12%. On days 2 and 3 the control tissues consumed oxygen at only 18 and 8%, respectively, of the initial rate, while those with T4 were maintained at 108 and 95%. After the first day's exposure to 5° there was little glucose increase in the controls but the vessels containing the T4 continued to produce glucose throughout the entire incubation period.

This series of carbohydrate determinations was carried out after each 37° incubation, and does not reveal how much of the T4 effect was exerted at 5° and how much at 37°. This point is examined more fully in Figure 3. The three sets of bars to the left show little if any T4 effect on oxygen consumption over 9 hours of incubation at 37°. At the same time, a steady glucose production took place in both blank and T4 vessels. Another set was analyzed directly after 48 hours of incubation at 5° without prior exposure to 37°. They reveal only a slight amount of gluconeogenesis, in both B1 and T4. During the next 3 hours at 37°, glucose production rose in both blank

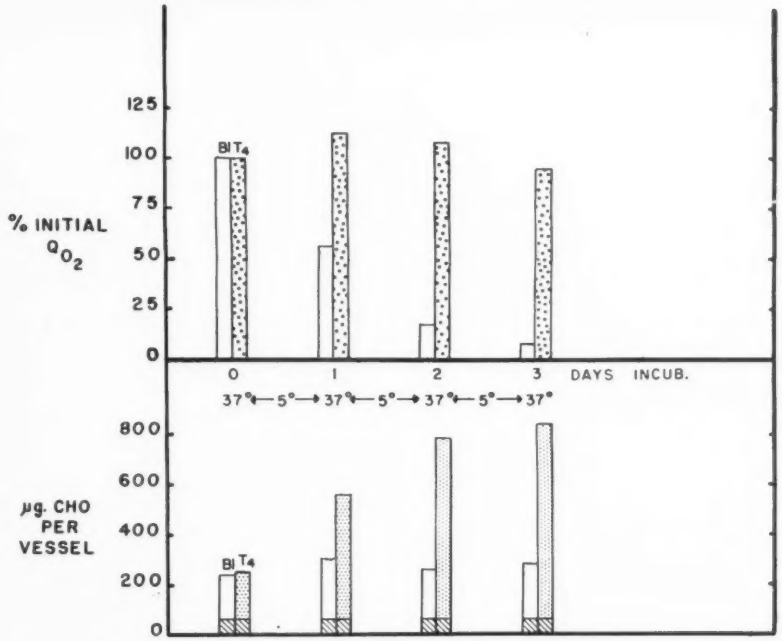

FIG. 2. Effect of thyroxine on the metabolism of kidney cortex incubated for 3 days in Ringer-18mM L-proline. The top bar graph compares the maintenance of initial Q_{O_2} between blank (B1) and thyroxine (T4) vessels. The lower graph represents the amount of glucose present in the blank and T4 vessels beyond that amount initially present in the tissue, shown by the cross-hatched lower portions. See text for complete explanation.

and T4, but only in the latter did it approach the 3-hour increment of vessels not exposed to 5°. While the control carbohydrate levels fell at 6 and 9 hours, T4 vessel values rose somewhat, but never to those reached during the continuous 37° incubation.

The oxygen consumption results were even more dramatic. In the T4 vessels, the Q_{O_2} was maintained at 105, 105 and 99% of the original initial Q_{O_2} at 3, 6 and 9 hours, respectively. This was in contrast to the rapid failure of the blanks, dropping from 58% at 3 hours to 19% at 9 hours. It thus seems probable that T4 must be protecting the enzyme systems involved in oxygen consumption as well as gluconeogenesis during storage at 5°. This deleterious effect of cold exposure only became apparent during the 37° incubation.

Effect of oxogenous glucose on metabolic rate and gluconeogenesis in the presence of proline (*Fig. 4*). After the usual 15 minute equilibration period, oxygen consumption was measured at 37° for 1 hour on the starting day and on each of 3 subsequent days of storage at 5°. It can be seen from the upper half of Figure 4 that the addition of 580 µg of glucose to the Ringer-

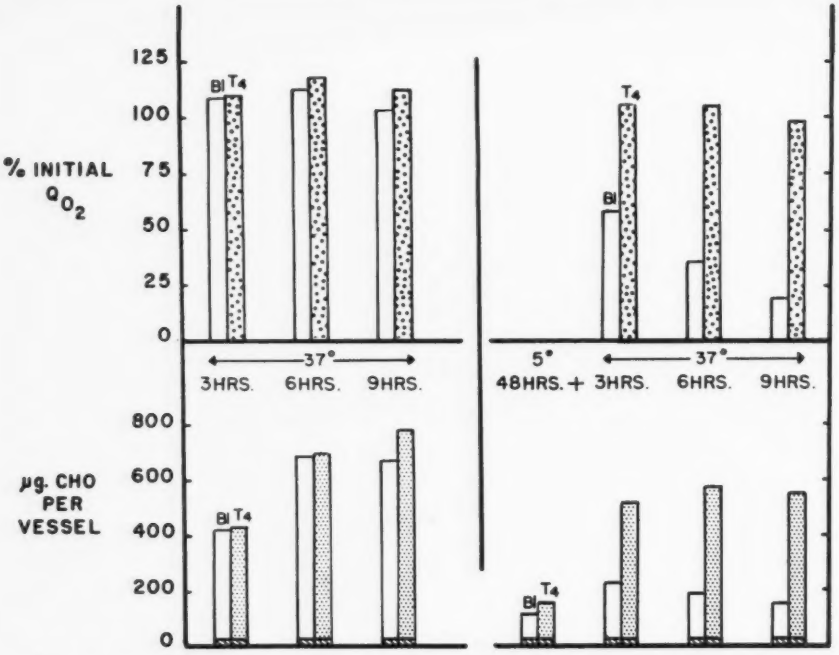

FIG. 3. Kidney cortex slice metabolism during 37° incubation immediately (left portion) or after preincubation at 5° for 48 hours (right portion). Oxygen consumption in blank (B1) and thyroxine (T4) vessels is above in each instance with corresponding glucose levels below. As before, the small cross-hatched part of each bar represents the carbohydrate initially present in the unincubated tissue.

proline solution from the beginning had little effect on respiration of either B1 or T4 tissue. However, carbohydrate analyses following the 37° incubation on Day 3 showed that this quantity of exogenous glucose depressed net glucose synthesis from proline by about 100 μg per vessel. A similar exogenous glucose depression of gluconeogenesis has been obtained during 6 hours of continuous incubation at 37° in the Ringer-proline medium. Percentage-wise, the decreased gluconeogenesis is far less in the T4 vessels.

Incubation at Constant Temperatures

Comparison of gluconeogenesis in proline, proline-glucose and glucose media at 5° (Fig. 5). In order to determine levels of gluconeogenesis attainable at 5°, vessels containing 3.0 ml. of Ringer-proline, Ringer-glucose (609 μg glucose per vessel) or Ringer-proline-glucose were kept at 5° for one to three days, without exposure to 37°.

There was an increased amount of glucose in all 3 media in both blank and T4 vessels. After 3 days, the tissue in proline plus T4 had produced

more glucose than the controls, regardless of the added glucose. The Ringer-glucose vessels still contained more glucose than the starting levels although less than on Day 1. The additional glucose was presumably produced from endogenous metabolites since tissue incubated in Ringer alone showed similar increases under other conditions (Figs. 1, 6).

Comparison of Ringer, glucose, L-proline and L-glutamate at 37°. As shown in the upper portion of Figure 6, 37° incubation of kidney cortex slices without added substrate (Ringer) resulted in a continuous drop in Q_{O_2} to less than 50% of the initial level by 9 hours. With 600 μg of added glucose, the initial Q_{O_2} was higher (see Fig. 1) and the falling-off was slightly less marked as shown at the upper right, Figure 6. Although no T4 effect was seen at $1\frac{1}{4}$ or 3 hours, there was a clearly evident action at 6 and 9 hours, quantitatively greater in the presence of glucose, but proportionally similar to that without added substrate.

The carbohydrate levels, in the lower half of Figure 6, were highest at $1\frac{1}{4}$

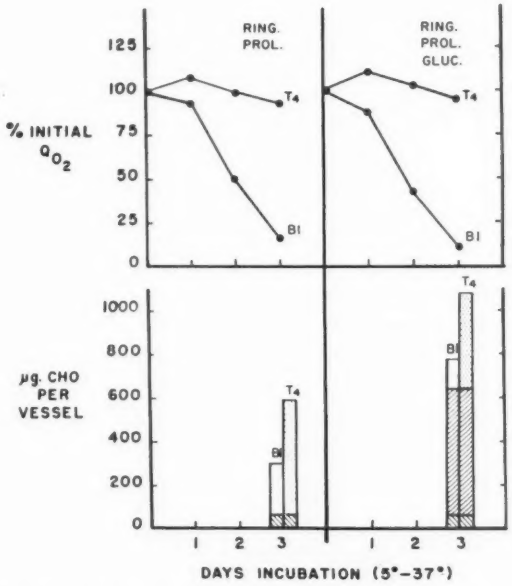

FIG. 4. Effect of exogenous glucose on kidney cortex respiration and production of glucose. In the upper graphs, maintenance of initial Q_{O_2} of the blank (B1) and thyroxine (T4) vessels in a Ringer-proline medium are compared over a 3-day period with those in a Ringer-proline-glucose medium (see text). The bar graphs below show carbohydrate levels after the three days at 5°, with the usual $1\frac{1}{4}$ hours exposure to 37° necessary for Q_{O_2} determinations on days 0, 1, 2 and 3. The lowest, cross-hatched portions show the initial tissue carbohydrate (52 μg/vessel) and the intermediate cross-hatched portions the 580 μg of glucose added per vessel. The plain (B1) and stippled (T4) portions represent gluconeogenesis in the appropriate vessels.

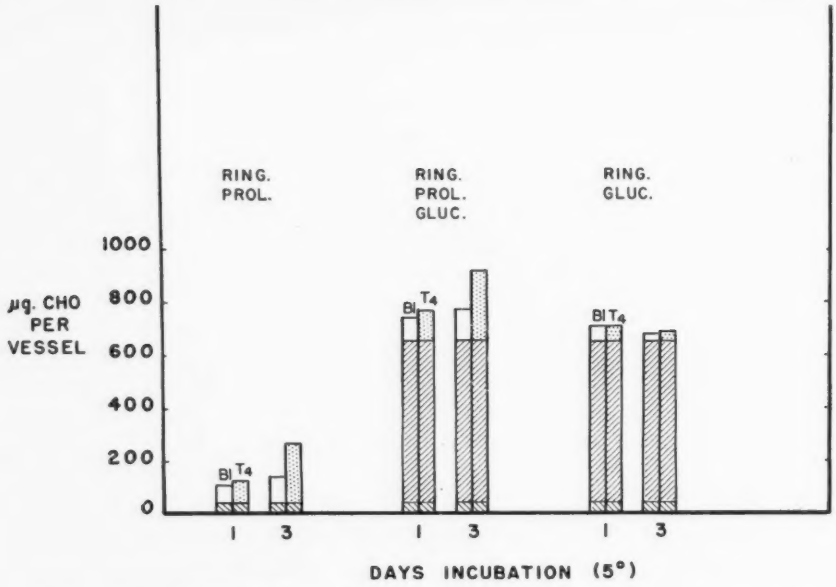

FIG. 5. Effect of thyroxine on gluconeogenesis by kidney tissue held at 5°. Carbohydrate levels of blank (B1) and thyroxine (T4) vessels are compared in three different media, Ringer-proline, Ringer-proline-glucose and Ringer-glucose. When added, exogenous glucose was 609 µg/vessel, shown as the larger, intermediate cross-hatched portions of the bars. The initial tissue carbohydrate level of 48 µg/vessel is represented by the lower cross-hatched portion.

hours, showing gluconeogenesis from endogenous precursors. Thereafter, the amount present decreased, indicating not only depletion of precursors, but a considerable utilization of the exogenous glucose. In no instance was there a significant T4 difference. The data in Figure 1 show that the initial Q_{O_2} was some 20% higher in Ringer-glucose over Ringer alone. In a 9-hour period the production of $C^{14}O_2$ from UC^{14} glucose accounted for a maximum of 18% of the oxygen consumed with oxidation of endogenous nitrogen-containing substrates accounting for an additional 35 to 45% (14). Thus, 35–45% of the endogenous oxygen consumption is unaccounted for and must be presumed to be at the expense of lipid.

In the presence of L-glutamate, the initial rate of respiration was 24% higher than with L-proline (Fig. 7), but the higher rate was not maintained beyond 3 hours. In the presence of proline, the Q_{O_2} increased to 120% of the initial by 6 hours and was above the initial level even after 12 hours. Although a difference in respiration between the control and T4 vessels had just appeared at 12 hours with proline, such a T4 effect was clearly present as early as 6 hours in the deteriorating glutamate system. The importance of a failing system for demonstration of a clear-cut *in vitro* T4 effect on

metabolism has been noted before, although its full significance is still a matter for conjecture.

Except for the 3 hour sample of the L-glutamate blank, glucose levels were consistently greater in the T4 vessels of both media.

Long term incubation in L-proline at 37° (*Fig. 8*). Oxygen consumption determinations between the 5th and 6th hours showed a 10% increase over the starting value, with the T4 tissue slightly ahead. As the metabolic rate fell steadily thereafter, the T4 superiority became more evident. At 18 hours, 0.165 ml. of 0.4 M proline solution was tipped in from the sidesac to restore the starting level of substrate, as determined in parallel experiments. After tipping, there was a marked upward swing in net glucose synthesis in the presence of T4, lasting about 6 hours. After a two-hour latency, the blanks showed a comparable rise, not maintained as well. The additional proline had little effect in changing the oxygen consumption of either B1 or T4.

The extra line extending from the 12 hour carbohydrate point shows the rate of disappearance of glucose from the Ringer-glucose flasks after 3 hours at 37° (from Fig. 6). Since the glucose levels were comparable, the somewhat slower rate of fall in the proline flasks suggests some continued gluconeogenesis. Considering the marked change of slope from 6 to 12 hours, it

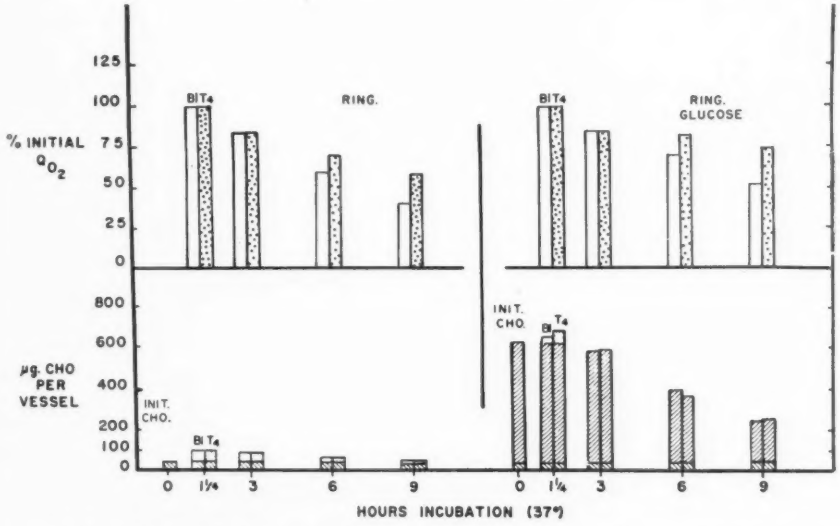

FIG. 6. Metabolism of kidney cortex slices during continuous 37° incubation in Ringer or Ringer-glucose medium. The Q_{O_2} values of the blank (B1) and thyroxine (T4) vessels are compared over a 9 hour period at 37° (top bar graphs). As before, the bottom cross-hatched portions represent the endogenous carbohydrate and the large cross-hatched portions the exogenous glucose, commencing with the initial addition of 600 μg/vessel. Net gluconeogenesis is shown by the plain or stippled bars for B1 or T4, respectively.

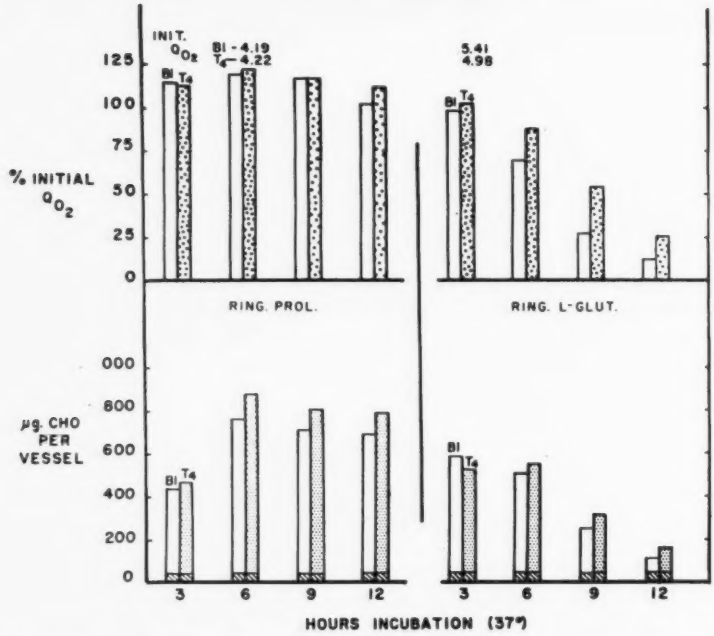

FIG. 7. Metabolism of kidney cortex slices with proline or glutamate as substrates. The initial Q_{O_2} and maintenance of blank (B1) and thyroxine (T4) are compared above. Accompanying carbohydrate changes are represented in the lower bar graphs. The initial tissue carbohydrate level is represented by the cross-hatched portion of each bar. Net gluconeogenesis by the blank (B1) and thyroxine (T4) vessels are shown by the plain and stippled bars, respectively.

is obvious that either (a) gluconeogenesis was extraordinarily rapid from 0–6 hours or (b) utilization of the new glucose was delayed for some unknown reason. In other experiments not shown here, we have tipped in additional proline at 6 hours. As in Figure 8, there was no change in Q_{O_2}, but glucose production continued to 9 hours without diminution and then showed its usual decline. This suggests that some enzyme system involved in the early rapid gluconeogenesis from proline must undergo failure after 6–9 hours at 37°.

DISCUSSION

The *in vitro* addition of thyroxine to thyroxine-deficient kidney cortex slices did not affect initial rates of oxygen consumption. Over extended periods of incubation, its addition maintained respiration at higher levels than the controls. During continuous incubation at 37°, differences between T4 and control oxygen consumption appeared at 6–9 hours and increased progressively with time. This respiratory effect of T4 was apparent even in

Ringer and Ringer-glucose media. In both, an initial net production of glucose occurred, soon followed by glucose diminution. The addition of alanine, proline or glutamic acid to the Ringer solution established a higher initial Q_{O_2} which continued for longer periods (except with glutamate) and increased net glucose. The further addition of T4 to each medium, particularly proline, maintained respiration and glucose at levels even higher than those obtained by the simple addition of the amino acid. With glucose as the sole substrate, the addition of thyroxine still produced better maintenance of oxygen consumption but did not affect the rate of glucose disappearance.

Incubation at 5° prior to that at 37° altered the metabolic picture by augmenting the differences seen. The mechanism of T4 in protecting against deterioration occurring in the cold is still unknown.

Thyroxine is undoubtedly affecting gluconeogenesis as well as respiration, for when the tissue was incubated in proline the greater the gluconeogenic effect the greater the respiratory effect. Exogenous glucose had little effect on respiration and the presence of T4 did not affect glucose disap-

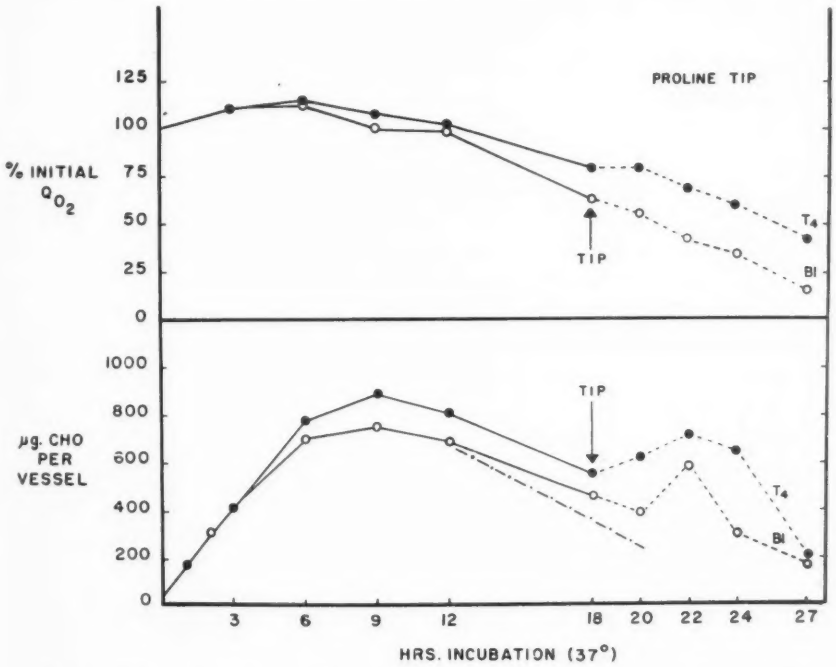

FIG. 8. Effect of tipping additional proline at 18 hours on metabolism of kidney cortex slices incubated at 37°. Oxygen consumption is shown in the upper half and glucose levels in the lower. As indicated, open circles are control vessels (B1) and solid circles (T4).

pearance. The rate of glucose oxidation was slow, at the most accounting for no more than 18% of the oxygen consumed.

Shipley (9) found that rat kidney cortex incubated in serum utilized no glucose and Sutherland (8) reported a decrease in oxidation of UC^{14}-glucose in the presence of acetate. Ammonia production from proline in our experiments was little affected by the addition of exogenous glucose as long as the respiration was well maintained (15). Glucose production was favored under optimal respiratory conditions and predominated during the first hours of incubation, regardless of substrate. Soskin and Levine (16) suggested that *in vivo* the kidney synthesized glucose from glucose fragments. Subsequently many investigators, studying organ function through various *in vivo* surgical procedures, found that the kidney was capable of forming glucose (17–20). More recently, renal A-V difference has indicated that the kidney produced 4 to 13% as much glucose as the liver (21). The absence of physilogical glycogen synthesis by the kidney, together with its limited glucose utilization undoubtedly forms the basis for its contribution to the blood sugar.

After 1 hour of incubation of kidney cortex slices at 37°, we have recovered 4% of the carbon from UC^{14}-glucose as $C^{14}O_2$ and after 9 hours 25% (unpublished results). These amounts would account for 11 and 17% of the oxygen consumed during the respective incubation periods. Serif and Wick (10) have also shown that labeled CO_2 was produced from kidney slices incubated in C^{14}-labeled glucose. Less than 1% of either the C_1 or C_6 labeled glucose appeared as $C^{14}O_2$ after 2 hours incubation. Accepting their figure of 150 μg glucose oxidized/gm. tissue/hour and assuming complete oxidation to CO_2 and a Q_{O_2} of 3.5 then only 3.2% of the oxygen consumed would be due to glucose oxidation. The observation (22) that slices of rabbit kidney cortex did not accumulate glucose against a concentration gradient has been explained as due to rapid utilization of the glucose. This hypothesis seems questionable, particularly since the incubation medium contained acetate, a compound which increases glucose production and decreases glucose uptake (8).

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THE EFFECT OF THE ADRENAL AND THYROID GLANDS ON ERYTHROCYTE GLYCOLYSIS[1]

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ABSTRACT

Thyroidectomized rats (14 to 26 days after operation) failed to show a marked change in erythrocyte glycolysis. Erythrocytes from similar rats maintained on 225 μg% thyroxine concentration in the drinking water exhibited a significant increase of 32%, whereas erythrocytes from normal animals given 1000 μg% thyroxine water exhibited a significant decrease of 25%. 3,5,3′-Triiodothyronine water at 108 μg% concentration given to normal and thyroidectomized rats, and thyroxine water at 225 and 500 μg% concentration given to normal rats produced no significant differences in erythrocyte glycolysis from the controls. Adrenalectomized rats, with or without daily cortisone injections (5 to 9 days), failed to show marked changes from control values for erythrocyte glucose metabolism. Normal rats treated similarly showed a significant decrease of 21%.

THE idea of using erythrocytes for studying the mechanism of hormone action is attractive since these cells can be isolated from the blood with relative ease for chemical and enzymatic analysis, can be studied at various stages of development, and have a distinct difference in metabolism between the mature and immature forms which may permit observations on either a predominantly anaerobic or aerobic metabolism. That mammalian erythrocytes may reflect hormone levels is suggested by their increased oxygen consumption when taken from hyperthyroid patients (1), or from thyroidectomized rats injected with thyroxine (2). However, the amounts of oxygen involved in studies on mature erythrocytes are so small that there is some question as to the significance of the respiratory metabolism. Since the mature erythrocyte is dependent almost entirely upon anaerobic glycolysis for its energy (3), and since thyroxine is capable of influencing anaerobic glycolysis in certain tissues (4), it seemed possible that significant changes in glucose metabolism might be observed in rat erythrocytes under various thyroid conditions. Macho (5) reported that blood from thyroidectomized rabbits showed a decreased glucose metabolism which could be reversed with thyroxine. However, no quantitative values for glucose utilization were given, nor any indication of the blood picture. More recently Calesnick, *et al.* (6) reported a decreased glucose metabolism

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in erythrocytes from rabbits given daily injections of triiodothyronine for 7 to 14 days. It had also been observed that a single dose of cortisone to a normal rabbit increased blood glycolysis for a period of 20 hours (7). There seems to be no literature concerning the effect on blood glycolysis if the animal is subjected to excess cortisone over a relatively long period of time.

The data presented here extend the observations on blood cell glycolysis to rat erythrocytes and are significant in showing that cortisone or thyroxine is capable of causing depression of glycolysis in these cells.

MATERIALS AND METHODS

Male albino rats purchased at 100 to 130 gm. were kept under laboratory conditions at least one week prior to use. The thyroid or adrenal glands were removed surgically under Nembutal-ether anesthesia. Either L-thyroxine sodium (Sigma) or 3,5,3′-triiodothyronine (Delta) was given in the drinking water to normal and thyroidectomized rats since large amounts of these substances in drinking water have been shown to increase total body metabolism of rats by 100% or more (8). The appropriate quantity of thyroxine or triiodothyronine was dissolved in 5 ml. of 0.02N. NaOH and diluted to 2 liters with water so that the final concentrations were either 225, 500, or 1000 μg% for thyroxine, or 108 μg% for triidothyronine. Fifteen mg. of cortisone acetate (Sigma) were dissolved in 2 ml. of methanol and diluted with saline to 15 ml. One ml. of this suspension was given subcutaneously to normal or adrenalectomized rats daily.

The importance of the nature of the bathing medium for the activity of thyroxine is indicated by the work of Barker (9) involving *in vitro* effects of thyroxine on the metabolism of kidney slices. Suspension of erythrocytes from treated and untreated adrenalectomized rats in a phosphate buffered solution may cause changes in cell volume (10) which could result in a dilution or concentration of cell contents. It has been estimated that erythrocytes account for 90 to 95% of the glycolysis in blood (11). Considering this and the fact that isolation and resuspension of erythrocytes result in a prolonged time interval before glucose utilization can be measured, it seemed pertinent to take blood from animals with minimum disturbance. Blood samples were drawn by cardiac puncture (no anesthesia) into a syringe rinsed with heparin-saline solution, and 2 ml. of the blood transferred to a glass stoppered flask, which was placed in a Warburg bath (37.5° C) and shaken at the rate of 90 cycles per minute with an amplitude of 3 cm. Preliminary experiments under these conditions with the glucose utilization measured at 30, 60, and 90 minute intervals showed a relatively constant glucose utilization. Thus duplicate samples were withdrawn from the flasks after a 5 minute equilibration period, and again 60 minutes later for glucose analysis by the Nelson-Somogyi method. After it was determined that no changes in hematocrit occurred during the incubation period, the hematocrit was determined in triplicate at the end of the period by using capillary tubing and centrifuging to constant volume. Red and white counts were made in duplicate or triplicate with a standard hemocytometer. The percent reticulocytes was determined in duplicate samples by alcoholic staining of dry smears with New Methylene Blue and Wright's stain.

RESULTS

Only 14 of the 152 rats used in this study showed a blood glucose concentration which deviated from the normal range of 80 to 120 mg.% at the first sampling after incubation. Such deviations occurred in no more than 1 to 3 samples in any one group. Careful inspection of the data failed to indicate a correlation between the initial glucose concentration and the amount

TABLE 1. THE EFFECTS OF THYROXINE, 3,5,3′-TRIIODOTHYRONINE, AND CORTISONE ON RAT BLOOD

Exptl. group[a]	No. of rats	Exptl. days[b]	Mg. glucose per hour[c]	Percent hemato-crit	RBC $\times 10^6$ per cmm	WBC $\times 10^2$ per cmm[d]	Percent reticulocytes[d]
Control	17		89 ± 6	43 ± 1	719 ± 21	98 ± 10 (8)	1.0 ± 0.4 (8)
Tx	19	22 (14–26)	82 ± 4	47 ± 1	877 ± 23	180 ± 17 (5)	2.3 ± 1.0 (5)
Tx + T_1	16	19 (16–24)	118 ± 9**	42 ± 2	717 ± 59	135 ± 15 (5)	1.5 ± 1.4 (5)
Normal + T1	14	15 (14–15)	74 ± 6	50 ± 1	1014 ± 23	143 ± 19 (9)	2.2 ± 0.6 (6)
Normal + T2	11	16 (14–17)	86 ± 5	47 ± 1	849 ± 27	123 ± 3 (6)	8.2 ± 1.3 (6)
Normal + T3	9	16 (14–17)	67 ± 7*	47 ± 1	900 ± 21	142 ± 17 (9)	1.9 ± 0.5 (9)
Tx + TIT	11	18 (14–21)	114 ± 14	46 ± 1	870 ± 48		
Normal + TIT	6	22 (21–23)	73 ± 5	48 ± 1	765 ± 43		
Adx	11	7 (5–8)	95 ± 9	45 ± 2	830 ± 28		
Adx + cortisone	13	8 (7–9)	97 ± 11	46 ± 1	819 ± 15	111 ± 12 (10)	3.4 ± 0.5 (10)
Normal + cortisone	13	7 (5–9)	70 ± 5**	49 ± 1	914 ± 26	86 ± 5 (11)	2.0 ± 0.9 (13)

[a] Tx = thyroidectomized. T1, T2, T3, = thyroxine water of 225, 500, and 1000 μg% concentration, respectively. TIT = 3,5,3,′-triiodothyronine water of 108 μg% concentration. Adx = adrenalectomized. Cortisone dosage of 1 mg./day/rat.

[b] The average number of days rounded to nearest figure with the range in parenthesis.

[c] Value for 100 ml packed cells. The ± in this and succeeding columns = standard error of the mean. $P < 0.05$ = *, $P < 0.025$ = **.

[d] The number in parenthesis refers to the number of rats represented in the sample.

of glucose utilized in the individual blood samples of any one group. There appeared to be no gross changes in the blood picture of the various groups in which cell counts were made to indicate any correlation of the cellular components with the glucose utilization. The mean values for hematocrit, erythrocyte count, white cell count, and reticulocyte percentage as indicated in Table 1, were within the normal range for rats (12). Therefore the glucose utilization by the blood from each rat was converted to mg of glucose utilized per 100 ml. of packed cells from the hematocrit. The number of animals, number of days of the experimental regime before taking blood samples, and the mean values for glucose utilization are presented in Table 1. Overnight fasting of a group of 12 normal rats failed to show a marked difference (−5%) in erythrocyte glucose utilization when compared to non-fasted rats (control group). All the data in Table 1 are from unfasted animals. The control group represents 10 unoperated and 7 sham operated rats which produced equivalent mean values; 88 and 90 mg./hr./100 ml. packed cells, respectively.

Removal of the thyroids had little effect upon erythrocyte glucose utilization (−7%). However, whether the thyroids were present or not appeared to influence the response to exogenous thyroid hormone. Cells from thyroidectomized rats treated with thyroxine water (225 μg%) responded with a 32% increase in glucose utilization which was significantly different from the control group ($P < 0.025$), whereas cells from normal animals exhibited either no change (500 μg%), or decreases of 17% (225 μg%) and 25% (1000 μg%). Only the highest concentration of thyroxine water produced a decrease significantly different from the control ($P < 0.05$). Although triidothyronine produced no statistically significant difference from the control, the glucose utilization did show a pattern similar to that produced by thyroxine; i.e., cells from thyroidectomized animals and normal animals showed an increase of 28% and a decrease of 18%, respectively.

Rats adrenalectomized for a period of from 5 to 9 days, with or without

cortisone treatment, failed to show significant differences in erythrocyte glucose utilization. However, normal rats given cortisone over a similar time interval showed a decrease of 21%, significantly different from the control ($P < 0.025$).

The fluid was noted in the thyroidectomized and normal groups given thyroxine water (225 μg%), and the normal group given triiodothyronine by measuring the amount of fluid remaining in the bottles when fresh thyroxine or triidothyronine water was prepared. Calculations based on the number of animals and the time intervals involved indicate that the intake was quite variable for the different groups in that they took approximately 23, 37, and 28 ml. of treated water per day per rat, respectively; or, in the same order, 52 and 85 μg of thyroxine and 30 μg of triiodothyronine.

The fact that the animals were not fasted prior to withdrawal of blood samples precludes a quantitative evaluation of weight changes. There were no indications of the development of toxicity from the thyroxine or triiodothyronine water in that the treated groups showed a mean weight gain of at least 10% per week. A similar gain (15%) was shown by the sham operated animals included in the control group. The adrenalectomized animals, treated and untreated, showed a mean weight gain of 10 and 13% for the period of 5 to 9 days. However, the normal cortisone treated group showed a mean weight gain of 60%.

DISCUSSION

The small decrease in glucose metabolism exhibited by the cells from the untreated thyroidectomized animals may have been due to lack of time for the full development of thyroid deficiency. That erythrocytes are affected by removal of the thyroid is evident from the difference in glucose metabolism produced by thyroxine or triiodothyronine in the presence and absence of the gland. Consumption of hormone by the thyroidectomized animal resulted in stimulation of glucose metabolism, but in the intact animal, treatment resulted in no change or a depression of erythrocyte metabolism.

As suggested by the data, the water intake of these animals could have been quite variable. Since no signs of toxicity or marked blook picture changes occurred, it is probable that hormone blood levels were minimal, and it is not surprising therefore that the group variations were not always significant in spite of the large mean changes in some of the groups. However, the results of this study tend to be supported by the observations that thyroidectomized rabbits injected with thyroxine exhibit an increase in blood glucose metabolism (5) and that erythrocytes from normal rabbits given daily intravenous injections of triiodothyronine over the same time intervals exhibit a decrease in glucose metabolism (6). It appears that the increase of total body metabolism in normal rats and thyroidectomized rats treated with thyroxine and triiodothyronine water (8) is not mirrored by erythrocyte glucose metabolism.

The data indicate that cortisone fails to influence the erythrocyte glucose

utilization unless the adrenal glands are present. Removal of the adrenal glands for 5 to 9 days failed to produce a significant change in erythrocyte glucose utilization whether cortisone was given or not, but cortisone given to normal animals resulted in a mean decrease in magnitude (21%) similar to that produced by thyroxine and triiodothyronine water. This depression in glucose metabolism is in contrast to the observation on rabbits where a single dose (50 mg./kg.) stimulated blood glycolysis for a period of 20 hours (7). The difference in these two circumstances may be due to the longer time interval involved in the present study.

In view of the observations that thyroxine can cause hypertrophy of the adrenal glands and affect the state of the cells of the anterior pituitary in rats (13), it is tempting to suggest the possibility that the depression in glucose utilization observed from treatment of normal animals may be due to pituitary-adrenal-thyroid relationship. The fact that the thyroids or adrenals must be present in order that thyroxine or cortisone produce a decrease in glucose metabolism suggests that this particular effect is not due only to action of the hormone directly on the blood cells but also to some unknown factor that may be common to both adrenal and thyroid. Since cortisone, as well as thyroxine and triiodothyronine, is capable of depressing erythrocyte glycolysis, the possibility of using this response as a clinical laboratory test for thyrotoxicity, as suggested by Calesnick (6), does not appear feasible. The metabolic variations in red cells under the conditions described here indicate they may be susceptible to analysis for elucidation of certain aspects of hormone action at a cellular level provided an experimental technique can be developed to obtain more consistent responses.

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HOMEOSTASIS OF ORGAN WEIGHT IN RESPONSE TO INCREASING DOSAGE OF TESTOSTERONE IN THE CASTRATED MOUSE[1]

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ABSTRACT

Castrated Strong A mice received 0.05, 0.1, 0.25, 0.5, 1.0 or 2.5 mg. of testosterone phenylacetate intraperitoneally every 10 days over a 40 day period. Thymic weights decreased, and seminal vesicle, kidney and submaxillary gland weights increased, over the dose range as a whole but showed no change between the 0.5 and 1.0 mg., or between the 0.25, 0.5 and 1.0 mg. dosages. Superficial lymph node weights exhibited no weight decrease between the 0.05, 0.1 and 0.25 mg. or between the 1.0 and 2.5 mg. dosages. These plateaus of non-response to increasing dosage, except the second one of node weight, occurred when the organ weights reached, or approached, those in untreated intact animals.

Other castrated Strong A mice received 0.25, 0.5, 1.0 or 2.5 mg. of the same androgen subcutaneously at identical intervals. The same plateaus in organ weights (except for the submaxillary glands) occurred at the same dosages as with intraperitoneal administration. At the same time, subcutaneous administration was less effective, at the lower dosages, than intraperitoneal in reducing node and increasing seminal vesicle weight, but more effective in reducing thymic and increasing kidney and submaxillary gland weight.

It appears that, in testosterone-sensitive organs, homeostasis occurs when the effect of exogenous androgen reaches the physiological level of endogenous androgen in the intact animal.

PRELIMINARY experiments indicated that the maintenance dose of testosterone phenylacetate,[2] administered subcutaneously to castrated mice of the Strong A strain, lay between 0.375 and 1.0 mg. every 10 days, judging by seminal vesicle and thymic weights; and that intraperitoneal administration was more effective than subcutaneous in increasing seminal vesicle weight and less effective in decreasing thymic weight (1). In contrast, a dose level of 7.5 mg. of the same androgen, also administered subcutaneously and at the same intervals, was required to inhibit the growth of transplants of hormonally-dependent adrenal cortical carcinomas in castrated hosts of certain strains of inbred mice; the same dosage did not

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[2] In this report "testosterone," unless otherwise specified, refers to testosterone phenylacetate in aqueous microcrystalline suspension of 50 mg./cc. (Perandren, Ciba).

affect the growth of the same neoplasms in their original hosts (2, 3, 4). The present study was undertaken to characterize the maintenance dose more closely and to compare the two routes of administration upon a wider spectrum of organs. From the results, it appears that the castrated mouse is able to adjust to a doubling, or even a quadrupling, of the minimal physiological (maintenance) dose without differing in response.

MATERIAL AND METHOD

Two hundred and forty male mice of the Strong A strain, from 3 to 5 months of age, were randomized into 12 groups of 20 animals each. The mice in 11 groups were then castrated. Six of these groups received, respectively, 0.05, 0.1, 0.25, 0.5, 1.0 or 2.5 mg. of testosterone intraperitoneally on the day of castration and 10, 20 and 30 days later. Four other castrated groups received 0.25, 0.5, 1.0 or 2.5 mg. subcutaneously at the same time. One castrated group and the non-castrated group received no treatment.

All animals were killed by chloroform on the fortieth day. At autopsy fresh weights were obtained, after careful blotting on filter paper, for the superficial lymph nodes, thymus, seminal vesicles, kidneys, submaxillary glands, adrenal glands, liver and spleen. The superficial nodes (5) comprised the right and left inguinals, weighed separately, and the duplicated medial superficial cervical nodes, those of each side being weighed as a unit. The thymus and submaxillary gland lobes, the seminal vesicles with contained fluid, the liver and the spleen were all weighed as single units. The right and left kidneys and adrenal glands were weighed separately. Thus, each experimental group provided individual weights for 40 single and 20 pairs of lymph nodes, 20 thymus glands, 20 seminal vesicles, 40 kidneys, 20 submaxillary glands, 40 adrenal glands, 20 livers and 20 spleens. Body weight for each animal was obtained immediately before death. At autopsy, depots of testosterone were grossly visible subcutaneously or intraperitoneally in animals in the groups receiving the 1.0 or 2.5 mg. dosages.

RESULTS

Mean weights, with their standard errors, were calculated for the total body and for each of the 8 organs[3] for all groups. These figures, plotted against a logarithmic scale of dosage, are shown for the groups receiving testosterone intraperitoneally or subcutaneously, with an indication of the means of the untreated intact and castrated groups, in Figures 1, 2, and 3. Probabilities of significance of difference between successive weights with increasing intraperitoneal or subcutaneous dosage, between all weights in the castrated groups and those in the intact group, and between weights for the same dosage via the intraperitoneal and subcutaneous route were determined.

Lymph nodes and thymus glands (Fig. 1).

These two organs showed decreasing weight with increasing dosage with an interposed plateau of non-response when the organ weight approximated that found in untreated intact animals.

[3] Mean organ weights, in the figures, represent 6 superficial lymph nodes, the whole thymus, both seminal vesicles, both submaxillary glands, one kidney, one adrenal gland, and the whole liver and spleen.

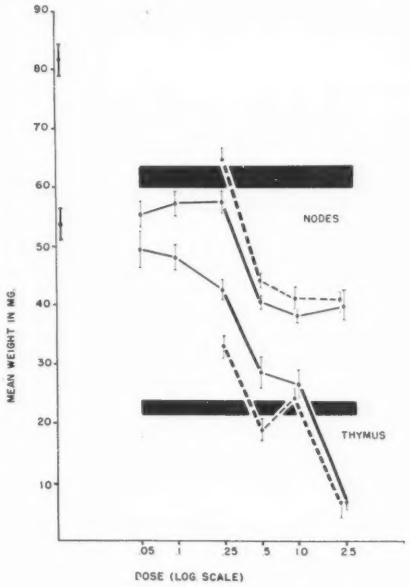

FIG. 1. Mean weights of the superficial lymph nodes and thymus gland in groups of 20 castrated male A strain mice, receiving various dosages (in mg) of testosterone. (Standard errors of the means are indicated by vertical lines; differences significant below the 5% level between successive means are indicated by the wide line and those not significant at this level by a narrow line joining the respective points; the mean and standard error for the corresponding organ in untreated intact mice of the same strain is indicated by a horizontal band and in untreated castrated mice by the isolated point; unbroken line—intraperitoneal; broken line —subcutaneous administration).

The pooled weights of the *6 superficial lymph nodes* showed a highly significant ($P < 0.001$) decrease between the 0.0 (untreated castrate) and 0.05 mg. dose levels of testosterone but no further decrease occurred at the 0.1 and 0.25 mg. dosages, administered intraperitoneally. Further increase of dosage to 0.5 mg. effected a second weight drop ($P < 0.001$), but there was no further change at the 1.0 and 2.5 mg. levels.

Subcutaneous administration of the steroid also produced a weight decrease between the 0.25 and 0.5 mg. dosages ($P < 0.001$), but no further change at 1.0 and 2.5 mg. There was no significant difference between node weights at the 0.1 and 0.25 mg. dosages and the node weight in untreated intact animals for either route of administration. There was a significant difference between these weights at the 0.05 mg. ($P < 0.05$) and at all other dose levels ($P < 0.001$).

Subcutaneous administration was less effective than intraperitoneal administration in producing weight reduction at the 0.25 mg. ($P < 0.001$) and at the 0.5 mg. dosage ($P < 0.05$), but there was no difference in weights with administration by either route at the higher dose levels.

Thymic weight was little affected by intraperitoneal doses of testosterone of 0.05, 0.1, or 0.25 mg.; no successive points on the weight-dose response line differed significantly, although those for the 0.0 and 0.25 levels did. A highly significant ($P < 0.001$) drop in weight occurred between the 0.25 and 0.5 mg. and again between the 1.0 and 2.5 mg. dosages. However, no appreciable weight decrease occurred between the 0.5 and 1.0 mg. dosages.

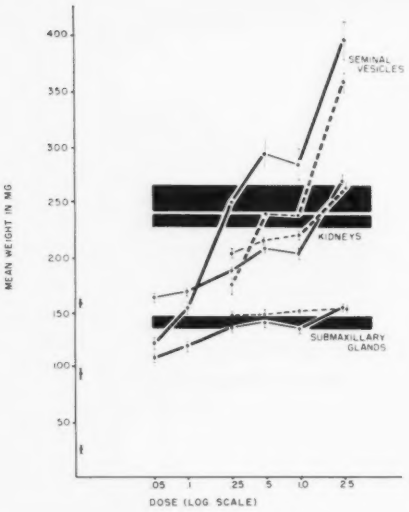

FIG. 2. Mean weights of the seminal vesicles, kidneys and submaxillary glands in groups of 20 castrated male A strain mice receiving various dosages (in mg.) of testosterone. (Symbols as in Figure 1.)

Subcutaneous administration, over the upper dose range (0.25 to 2.5 mg.) gave a response curve parallel to that for intraperitoneal administration; again, there was no significant change between the 0.5 and 1.0 mg. dose levels, but there was ($P < 0.001$) between the 0.25 and 0.5 mg. and between the 1.0 and 2.5 mg. dosages. All thymic weights differed ($P < 0.001$) from the weight in untreated intact animals except those at the 0.5 and 1.0 mg. dose levels for both routes of administration.

There was a significant difference between the thymic weights with intraperitoneal or subcutaneous administration at the 0.25 ($P < 0.01$) and 0.5 mg. ($P < 0.05$) dosages, with the latter route effecting a greater weight reduction than the former. At the higher dosages this differential disappeared.

Seminal vesicles, kidneys and submaxillary glands (Fig. 2)

These organs showed increasing weight with increasing testosterone dosage with an interposed plateau of non-response when the organ weight approximated (seminal vesicles and submaxillary glands) or approached (kidneys) that found in untreated intact animals.

The weights of the *seminal vesicles* showed a significant difference($P < 0.05$ or < 0.01) between all dose levels, except 0.5 and 1.0 mg. The weights did not differ significantly from those in untreated intact animals at 0.25 and 1.0 mg. but did at the intermediate level of 0.5 mg. ($P < 0.05$) and at all other dosages ($P < 0.001$).

Subcutaneous administration also produced differences ($P < 0.01$) between successive seminal vesicle weights at 0.25 and 0.5 mg., and again between those at 1.0 and 2.5 mg., but there was no change between the

weights at 0.5 and 1.0 mg. At these two latter dose levels the organ weights in castrated animals did not differ significantly from those in untreated intact animals.

Subcutaneous administration was less effective ($P < 0.01$) in increasing seminal vesicle weight than intraperitoneal at all dose levels except the highest (2.5 mg.).

The weight-dose response of the kidneys to intraperitoneal administration, except for their lesser responsiveness at the lower dose levels, resembled that of the seminal vesicles. There was no appreciable weight increase between the 0.0 and 0.05 mg., or between the 0.05 and 0.1 mg. dosages but a highly significant one ($P < 0.001$) between the 0.1 and 0.25 mg. and between the 0.25 and 0.5 mg. levels. There was no significant change in weight between the 0.5 and 1.0 mg. dosages, but there was between the 1.0 and 2.5 mg. levels ($P < 0.01$). However, unlike the seminal vesicles, all kidney weights differed ($P < 0.001$) from those of the untreated intact animals.

Subcutaneous administration produced no appreciable weight increase in the kidneys between the 0.25 and 0.5 mg., or between the 0.5 and 1.0 mg. dosages but did between the 1.0 and 2.5 mg. levels ($P < 0.001$). Again, unlike the seminal vesicles, all weights differed ($P < 0.05$ or < 0.001) from those in untreated intact animals.

The effectiveness of subcutaneous administration on increasing kidney weight was greater than that of intraperitoneal administration at the 0.25 and 1.0 mg. dosages ($P < 0.01$ or < 0.001) but not at the other two levels (0.5 and 2.5 mg.).

The weight-dose response of the *submaxillary glands* was less marked than either that of the seminal vesicles or the kidneys. None-the-less, all successive points for increasing intraperitoneal dosage of testosterone differed ($P < 0.05$, < 0.01, or < 0.001), except those between the 0.25 and 0.5 mg., and 0.5 and 1.0 mg. levels. The weights at these dosages also did not differ from those in untreated intact animals.

Subcutaneous administration produced no significant weight change at any successive dose; the weights of the submaxillary glands only differed ($P < 0.01$) from these in untreated intact animals at the 2.5 mg. dosage. Subcutaneous administration, as in the case of the kidneys, was more effective than intraperitoneal in increasing gland weight at the 0.25 and 1.0 mg. dose levels ($P < 0.05$ or < 0.01) but produced no difference at the 0.5 or 2.5 mg. levels.

Adrenal glands, spleen, liver and total body weight (Fig. 3)

These three organs, and the whole body, exhibited no consistent response to increasing testosterone dosage. With the adrenal glands, there appeared to be a trend of weight reduction with subcutaneous administration, in contrast to fluctuations around the mean with intraperitoneal administra-

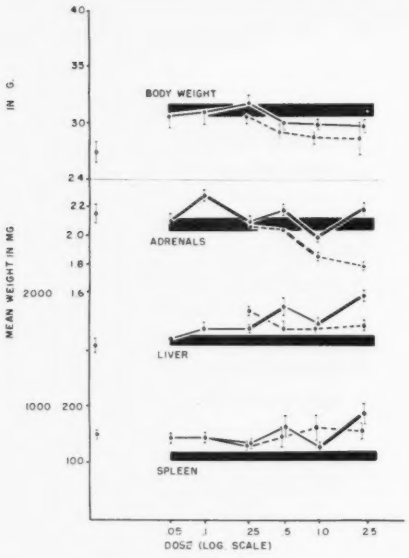

FIGURE 3. Mean weights of the whole body, and of the adrenal glands, liver and spleen in groups of 20 castrated male A strain mice, receiving various dosages (in mg.) of testosterone. (Symbols as in Figure 1.)

tion. Spleen weights tended to be greater in castrated animals receiving testosterone than in untreated intact animals. Total body weight appeared to increase with the lower dosages and to decrease at the higher dosages, but the only significant differences were between the weight of intact untreated animals and untreated castrated animals ($P < 0.01$) or castrated animals receiving 1.0 mg. subcutaneously ($P < 0.001$).

DISCUSSION

It seems probable that the action of testosterone upon the lymph nodes, thymus, kidneys and submaxillary glands in the mouse is selective, resembling that upon the seminal vesicles, rather than being part of a general protein-anabolic reaction or mediated by pituitary hormones. Both the kidneys and submaxillary glands of the mouse have been shown to have sex-specific histological differences (6, 7). The dose-response curves for all of these organs were generally similar; the maintenance dose for all, except the nodes, fell within the range of 0.25 to 1.0 mg. of testosterone every 10 days. A dosage of 100 μg daily of testosterone propionate has been considered to be above the physiological level (8); the same dosage did not increase seminal vesicle weight appreciably more than did 50 μg (9). A similar dosage was also thought to be above maintenance level by other workers (10). All of these dosages of testosterone propionate were applied to castrated rats; they fall in the same range found here for testosterone phenylacetate in the castrated mouse.

The most striking observation was that there was virtually no additional

response of the seminal vesicles and submaxillary glands when the dose was increased from 0.25 to 1.0 mg., or of the kidneys and thymus from 0.05 to 1.0 mg., despite the fact that highly significant responses occurred with change of dosage above or below these. Further, this failure to respond occurred when the weight of the organ reached (seminal vesicles, submaxillary glands and thymus gland), or approached (kidneys), the range found in untreated intact animals. In other words, when exogenous androgen maintained sensitive organs in castrated animals at a weight equal to that induced by endogenous androgen in intact animals, a phase of homeostasis occurred. Adjustment to increased androgen was effected, over a doubling or quadrupling of dosage, possibly by increased metabolism from enzymatic adaptation, or possibly by the simple reaching of a renal threshold. Further dose increase, evidently, overwhelmed the homeostatic mechanism and sensitive organs responded beyond the normal physiological limits.

The response of the superficial lymph nodes resembled and differed from that of the other organs. Firstly, the plateau of non-response occurred at a very low dose range (0.05 to 0.25 mg.), and, secondly another plateau occurred over the 0.5 to 2.5 mg. range. This second plateau may have represented a maximal response, beyond which the nodes were refractory to increased testosterone; if so, such a point was not reached with the other organs. The response of nodes, thymus, and spleen to hormones has been shown to differ by several authors. Marder (11, 12) found that thyroxine increased node and splenic weights but did not affect that of the thymus in the mouse; Money, Fager and Rawson (13) stated that testosterone decreased thymic but increased nodal weight in rats; and Santisteban (14, 15) showed that thymic, but not nodal, involution was produced by alcohol-induced stress, unlike the involution given by hydrocortisone.

That the homeostatic effect was not an artefact is suggested by the facts that, above and below the plateau, highly significant weight changes were produced by alterations of dosage; that the same plateaus occurred in different experimental groups receiving testosterone either intraperitoneally or subcutaneously at the same dose level; that the seminal vesicles and submaxillary glands showed a plateau with a wider range than that for the thymus and kidneys and that the plateau had an entirely different range for the superficial lymph nodes; and that similar plateaus were not exhibited by total body weight, or by organ weight for the liver, spleen and adrenal glands.

In organs that were sensitive to testosterone, intraperitoneal and subcutaneous administration differed in effectiveness at the lower dosages. Intraperitoneal, in contrast to subcutaneous, administration of androgen was found to be ineffective in the rat (16), or relatively ineffective until high dosages were reached in the capon (17). It was suggested that the steroid, given intraperitoneally, was largely absorbed via the hepatic portal venous system and was then inactivated by the liver. Such absorption could only

be partial for the peritoneal lining of the abdominal cavity, as distinct from the peritoneum covering the gut and forming the mesenteries, has systemic venous drainage. Further, in the present study, differences in response to intraperitoneal and subcutaneous administration were not consistent. The former was more effective in increasing seminal vesicle and decreasing lymph node weights than the latter, yet less effective in reducing thymic and raising kidney weights. In the experiments of Leathem (16) and Bernstorf (17) oily solutions of testosterone propionate were used for the subcutaneous and intraperitoneal routes and the vehicle, rather than the route *per se*, may have been critical. On the other hand, testosterone phenylacetate has been shown to be the most effective of a series of androgens, both in increasing seminal vesicle weight and in the duration of its effect (18).

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THE EFFECT OF ESTROGEN ON PLASMA LUTEINIZING HORMONE (LH) ACTIVITY IN THE RAT[1]

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ABSTRACT

Using the ovarian ascorbic acid assay for LH, detectable quantities of LH were found in plasma of ovariectomized donor rats. The LH activity of ovariectomized rat plasma was decreased by single subcutaneous injections of estradiol benzoate (Eb). This effect was demonstrable within a day after injection of a large dose of Eb (50 μg); the minimal effective dose of Eb to cause a decrease in LH activity 3 days after injection was about 0.1 μg. A slight decrease in LH activity was demonstrable within an hour of intravenous injection of a large dose of estradiol (E) or Eb. Decreased LH activity was found in the absence of proestrous or estrous smears but was always found if the smear was in proestrous or estrous stage. It was concluded that E exerts a negative feed-back on the LH controlling area(s).

THE effects of estrogen on the secretion of gonadotrophins by the adenohypophysis are complex (1). This steroid appears to have a dual capability of stimulating and inhibiting gonadotrophin secretion. For example, estrogens can inhibit ovarian development in the immature rat by suppressing gonadotrophin release (2, 3, 4), whereas, on the other hand, injection of estrogen can induce ovulation in the pregnant rat (5) and can advance ovulation in the cyclic rat (6), observations interpreted to mean that a release of LH has occurred. Ovariectomy, on the other hand, with removal of both ovarian steroids from the circulation leads to elevated levels of LH in both plasma and pars distalis, suggesting that removal of negative feed back from ovarian steroids leads to increased rates of synthesis and secretion of LH (6, 7).

In the present study the effects of single injections of estrogens on the plasma LH activity of ovariectomized rats have been evaluated using the most sensitive assay for LH yet devised, the ovarian ascorbic acid depletion method of Parlow (8).

METHODS

All rats were of the Sherman strain and were housed in a constant temperature room (80° F) with twelve hours of light and darkness per day. Donor rats were adult virgin females which had been ovariectomized 3–16 weeks. These donors were injected with

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steroids subcutaneously or intravenously, or received no injection and served as controls. For subcutaneous injections, estradiol benzoate (1 mg./ml.) was appropriately diluted with sesame oil, such that the injection volume was always 0.1 ml. For intravenous injections, estradiol or estradiol benzoate was dissolved in 95% ethanol and diluted with 0.9% saline, such that 0.15 ml. of a 20% alcoholic solution was injected intravenously while the rats were anesthetized with ether.

At the proper time following injection of steroids, the donor rats were anesthetized with ether and bled from the external jugular vein. Four to six ml. of blood were collected in 1–3 minutes in a syringe moistened with 0.1 ml. of heparin (1000 U./ml.). Blood from four donors which had been similarly treated was pooled and the plasma separated by centrifugation. The pooled plasma sample of 10–12 ml. was tested by the ovarian ascorbic acid depletion method in immature rats pre-treated with gonadotrophins (9). Following removal of one ovary for a control value of ovarian ascorbic acid, two ml. of plasma were administered intravenously over a period of 45–60 seconds to each assay rat. One hour later the second ovary was removed, and its ascorbic acid concentration determined. The percentage depletion (decrease) in ovarian ascorbic acid concentration in the second gland as compared to the first constituted the response taken as an index of the LH activity of the plasma (6, 9). In presenting the results (Fig. 1–2, Tables 1 and 2), the mean value of ovarian ascorbic acid depletion obtained on assay of 1–4 pools of plasma from rats subjected to each type of treatment schedule is presented. Significance of differences from the control ovariectomized value were calculated using Student's t test.

RESULTS

The Effect of Ovariectomy on Plasma LH Activity. Previous work indicated that 2 ml. of plasma from normal females possesses insufficient LH activity to deplete ovarian ascorbic acid of the recipient assay rats, and that detectable LH activity is found within a week following ovariectomy (7). This elevated plasma LH activity is maintained without significant increment for at least 16 weeks after spaying (7). The mean ovarian ascorbic acid depletion obtained on testing each of ten pools of plasma is presented in Table 1. The LH activity found in ovariectomized rats was compared with that found after injection of ovarian steroids (Table 2, Figs. 1–2).

TABLE 1. OVARIAN ASCORBIC ACID DEPLETION INDUCED BY PLASMA FROM OVARIECTOMIZED RATS

Plasma pool number	Time, post ovariectomy (weeks)	% ovarian ascorbic acid depletion of recipient rats
1	16	13.3 ± 3.5 (5)[1]
2	6	19.9 ± 2.5 (5)
3	6	16.6 ± 2.4 (6)
4	6	18.0 ± 2.3 (5)
5	4	10.2 ± 2.7 (5)
6	4	16.3 ± 1.3 (5)
7	4	15.9 ± 2.8 (5)
8	4	12.3 ± 2.0 (5)
9	4	12.5 ± 1.5 (6)
10	4	14.7 ± 3.7 (5)
Overall Mean		15.3 ± 0.8 (52)

[1] Mean ± SEM; no. of assay rats in parenthesis.

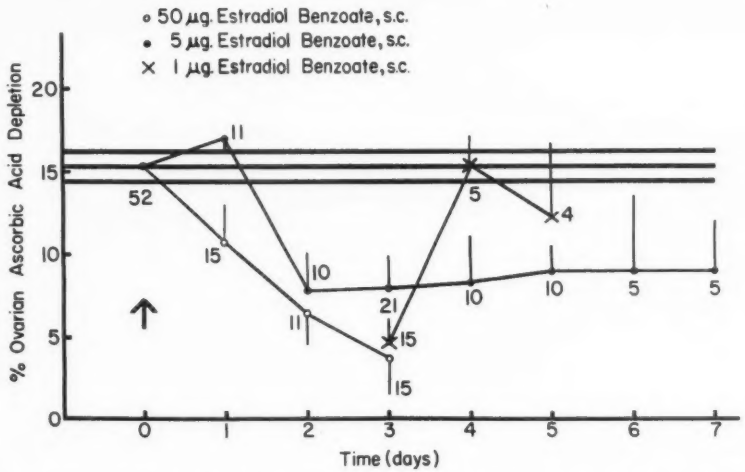

FIG. 1. The effect of subcutaneous injections of estradiol benzoate on the LH activity of ovariectomized rat plasma. The three horizontal lines near the top of the figure represent the values found in the ovariectomized controls. The line in the middle represents the mean value, the lines above and below this correspond to plus and minus one standard error of the mean. The vertical line at each point represents one standard error of the mean, while the numbers at the points refer to the number of assay rats employed to obtain the mean value. The arrow refers to the time of injection of steroid.

The Effect of Subcutaneous Injection of Estradiol benzoate (Eb) on Plasma LH Activity. Injection of the massive dose of 50 µg of Eb produced a linear decrease in LH activity with time (Fig. 1); a detectable decline in activity was already present when tests were first made one day following injection ($P < 0.02$), and a minimal value was found three days after the injection ($P < 0.001$). At this time there was no detectable LH activity present. Five µg of Eb similarly led to decreases in LH activity on measurement two and three days after injection of the steroid ($P < 0.001$ at both times), but in this case no appreciable depression of activity was apparent one day after injection. LH activity failed to return to the pre-injection value for at least a week. One µg of Eb also produced a marked suppression of LH activity on assay three days later ($P < 0.001$), but in this case the activity returned to the pre-injection level by the fourth day. The minimal effective dose of Eb required to suppress LH activity appeared to be of the order of 0.1 µg, when activity was estimated three days after injection of the steroid (Table 2).

Vaginal smears were examined in the ovariectomized donors to see if the effects of Eb on LH activity could be correlated with the development of vaginal estrus. In general, following Eb injection, depressed levels of LH activity were found to coincide with estrous or proestrous smears; however, decreased levels were also found after injection of the 5 µg dose on days 5, 6

TABLE 2. THE EFFECT OF VARIOUS DOSES OF ESTRADIOL BENZOATE (Eb) ON PLASMA LH ACTIVITY THREE DAYS POST-INJECTION

Dose of Eb (μg)	% ovarian ascorbic acid depletion	P vs. control
0 (Ovariectomized Controls)	15.3 ±0.7 (53)	—
0.1	9.6 ±1.7 (10)	<.01
0.2	6.0 ±1.7 (5)	<.01
1.0	4.7 ±1.5 (15)	<.001
5.0	8.2 ±1.9 (20)	<.001
50.0	3.7 ±2.2 (15)	<.001

All values: Mean ±SEM; no. of assay rats in parentheses.

and 7 when the smear had returned to the diestrous condition, and after the 0.2 and 0.1 μg doses on day three while the smears were diestrus. The presence of an estrous or proestrous smear, however, was always associated with a decrease in LH activity in plasma.

The Effect of Intravenous Injection of Estradiol (E) on Plasma LH Activity. E or Eb was also given by intravenous injection to see if this method of administration would result in a shorter latency of action (Fig. 2). Within an hour after injection of either substance by this route the plasma LH activity was slightly but significantly lowered ($P < 0.001$ in each case). The

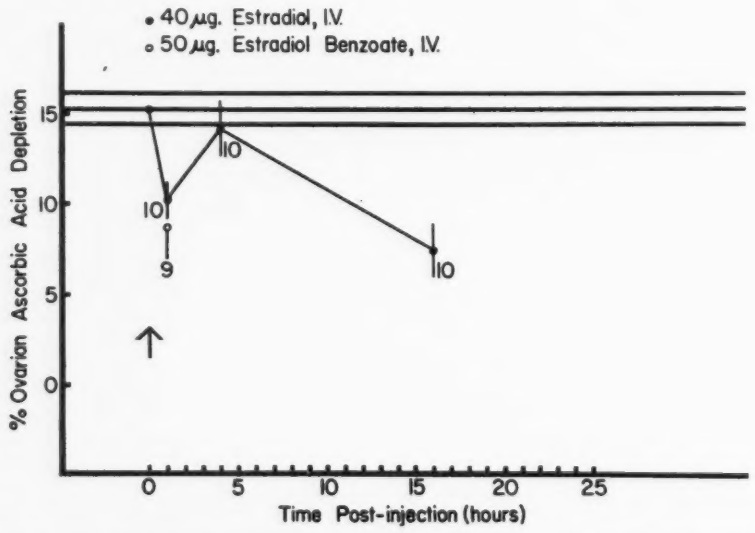

FIG. 2. The effect of intravenous injection of estrogen on the LH activity of ovariectomized rat plasma. The three horizontal lines near the top of the figure represent the values found in the ovariectomized controls. The line in the middle represents the mean value, the lines above and below this correspond to plus and minus one standard error of the mean. The vertical line at each point represents plus or minus one standard error of the mean, while the numbers at the points refer to the number of assay rats employed to obtain the mean value. The arrow refers to the time of injection of steroid.

mean ovarian ascorbic acid depletions induced by the individual pools of plasma were 10.3 ± 1.6 (5)[2] and 10.0 ± 1.6 (5)[2] in the case of E and 7.3 ± 3.2 (4)[2] and 10.0 ± 1.9 (5)[2] in the case of Eb. Yet a significant activity was still present at this time, and surprisingly, the activity returned to the pre-injection level 4 hours after injection of E. Since the rise occurring at 4 hours was small, it may not be a real phenomenon. The activity was markedly depressed on assay at 16 hours ($P < 0.001$). Vaginal estrus was still absent at this time.

DISCUSSION

The present results clearly show that estrogen in large or small doses is able to decrease LH activity in plasma of ovariectomized rats, presumably because LH secretion is markedly inhibited by estrogen. A minimal effect can come on within one hour following intravenous injection and is pronounced 16 hours after either intravenous or subcutaneous routes of administration of large doses. The latency of this effect is less than the time required for development of a proestrous or estrous vaginal smear, but appears to be longer than that of corticoids in suppressing the release of ACTH. In this latter case, Sydnor (10) reported suppression of ACTH secretion within 4 minutes of injection of hydrocortisone. The time required for disappearance of LH from the circulation appears to be too short to account for this difference between our results and those of Sydnor. (The half-life of exogenous LH in the circulation is of the order of 5–15 minutes (Taleisnik and McCann, unpublished, 11).) We believe that stress incident to the injection probably does not influence these values since various stresses, such as formalin injection, injection of Pitressin and prolonged etherization have had only minor effects on plasma LH activity (McCann and Taleisnik, unpublished).

Since removal of ovarian steroids results in elevated levels of LH in plasma (6, 7) which are decreased by administration of estrogen, the present results are in harmony with the concept that this steroid regulates LH secretion by negative feed-back on the LH controlling area(s).

Ganz has recently reported that estradiol given for one month can suppress the elevated serum LH found in spayed females using the ventral prostate assay (12). His results are in agreement with ours in this respect, but he also reported detectable activity in normal serum. This latter result is difficult to correlate with our findings since we have found no activity in normal rat plasma using an assay which is much more sensitive than the ventral prostate technique.

As stated earlier, there is a large body of evidence indicating that estrogen can also facilitate LH secretion under certain circumstances (1). No evidence for stimulation of LH secretion was found, but the present results

[2] Mean ± standard error of mean (no. of assay rats tested).

do not negate such a possibility. Perhaps with different permutations and combinations of estrogen-progesterone dosage such an action will be demonstrated.

Acknowledgments

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STEROID-INDUCED CONVULSIONS IN EXPERIMENTAL ANIMALS[1]

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ABSTRACT

High doses of Reichstein's Cpd. S cause tonic-clonic convulsions in rats and cats.

Both the clinical and EEG data clearly indicate that limbic structures are predominantly involved in these convulsions. The progression of the discharges is always initiated in the hippocampus.

Possible mechanisms for these seizures are discussed which may be related to the development of convulsions in man.

INTRODUCTION

A LARGE series of naturally-occurring and synthetic steroids have been reported as possessing anesthetic properties in animals (1, 2, 3, 4), and one of them (hydroxydione)[3] is now used as an anesthetic agent in surgery (5). Recently (6, 7) attention was called to the fact that some naturally occurring steroids (e.g. androstenolone,[4] Riechstein's Cpd. S)[5] were, after a single injection, capable of inducing generalized convulsions in rats. These convulsant effects have received far less attention than the anesthetic properties of other steroids.

This report deals with experimental data obtained from cats and rats. We shall describe the clinical and electrographic aspects of seizures induced by Cpd. S (Reichstein).

METHODS

Young Wistar albino female rats, weighing 50–60 gm., were employed in the initial phase of the experiments. This involved observation of the clinical changes induced by the steroid, and testing of drugs expected to antagonize or potentiate them.

Young cats of either sex, weighing 1.5–2.0 kg. were used for electrical recording. Both acute and chronically implanted preparations were employed.

In acute, terminal experiments the procedure included tracheal intubation and stereotactic placement of electrodes under ether anesthesia. These were followed by im-

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[2] Kenny Foundation Scholar.

[3] Pregnane-3,20-dione-21-ol sodium hemisuccinate.

[4] Synonyms: Δ^5-dehydro-iso-androsterone, Δ^5-dehydro-epi-androsterone.

[5] = 17α-hydroxy-11-desoxycorticosterone, also named cortexolone.

mobilization with a curariform drug (Flaxedil®) and frequent procainization of all wound margins and pressure points during the remainder of the experiment. The animals were artificially ventilated under positive pressure. Electrical recording was started at least two hours after discontinuation of the ether. All records were taken between focal leads and a common reference in the skin. In some experiments, a pair of steel needle electrodes, spaced vertically at a distance of 1 mm., was employed to record simultaneously from two levels in the dorsal hippocampus.

In chronic experiments, steel needle electrodes were stereotactically implanted in multiple subcortical loci. Cortical silver ball electrodes were placed on the intact dura. The whole assembly was joined to the skull and to a multi-lead miniature connector with dental cement. This implanting procedure was carried out under pentobarbital narcosis. Electrical records were taken 2–3 weeks later by connecting the animals to an eight-channel ink-writing oscillograph through a flexible multi-lead cable.

In all illustrations, negativity at the focal leads is shown as an upward deflection of the traces.

All deep electrode placements were controlled in serial sections of the formalin-fixed brains.

Cpd. S (Reichstein) was obtained as the free alcohol, the acetate or the succinate (=hemisuccinate). The free alcohol and the acetate were suspended in heated sesame oil for intraperitoneal injection. The succinate was dissolved in water with one molar equivalent of sodium carbonate. This solution, too, was injected intraperitoneally. All other drugs were dissolved in normal saline and injected by the same route.

RESULTS

Clinical effects of Cpd. S in rats and cats

After a dose of 300–400 mg./kg. of the steroid succinate the outstanding feature, in both cats and rats, was the development of generalized tonic-clonic convulsions. These occurred first about 15–35 min. after i.p. injection. They lasted up to one minute and re-appeared at progressively shorter intervals until the animal died or was treated with general anesthetics. As time progressed the animals became increasingly depressed in the intervals between seizures. Finally status epilepticus with decerebrate rigidity and tonic seizure activity developed. When Cpd. S (free alcohol or acetate) was given in oil (at a dose of 700–900 mg./kg.) seizure activity first appeared 1–3 hours after injection and lasted for many hours (up to more than 12 hours in some animals). In both species the character and time course of the grand mal seizures was similar.

The period preceding the development of the convulsions was characterized by quickly increasing inactivity in rats while agitation developed in cats. This period started 5–15 min. after injection of the steroid (in water or in oil). Cats showed marked tachypnea, sialorrhea, mydriasis and usually retching, with or without vomiting. Often this agitated phase in cats was accompanied by aimless searching movements or interrupted by "freezing" and staring. By contrast, rats only became tachypneic and, in addition, showed the following typical signs during the pre-seizure period: They often sat up and rubbed their snouts with both fore-paws. The paws became exceedingly sensitive to touch as judged by withdrawal movements

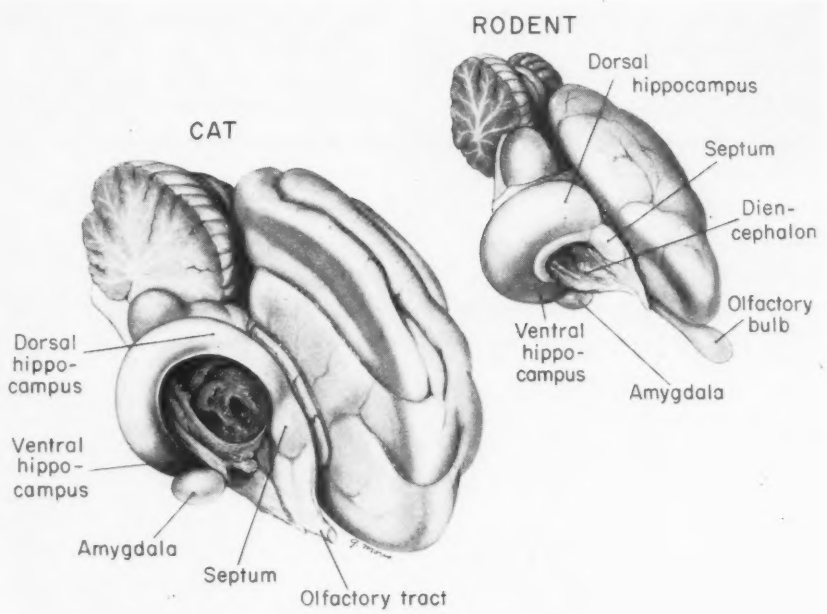

FIG. 1. Gross anatomy of the hippocampus and amygdala of the cat and rodent.

and screaming. This was followed by exophthalmos, twitching of face and ears, and a peculiar movement of some animals in which they backed up a few steps with their hind legs set far apart. Shortly afterwards, the grand mal seizures occurred.

Electrographic changes induced by Cpd. S in cats

Electrodes had been placed, in all preparations, in the midbrain tegmentum, thalamus (VPL), lateral hypothalamus, dorsal hippocampus (Fig. 1), amygdaloid and caudate nuclei, and sigmoid, suprasylvian and posterior marginal cortex.

After an intraperitoneal dose of 100–400 mg./kg. of Cpd. S scucinate (or of the free alcohol or acetate in oil at a higher dose level) characteristic recordings were obtained in both acute and chronic preparations.

In all freely moving "chronic" cats (Fig. 2B), but also (to a much lesser degree) in "acute" preparations, a rhythmic, high frequency high-amplitude pattern was recorded from the amygdala. This was the first significant electrographic change and coincided with the pre-seizure behavior described in the preceding section. Similar amygdaloid patterns were reported after cocaine (8) and in conditions of excited attention (9) in cats.

Following this, abnormal rapid, high-voltage discharge, later breaking

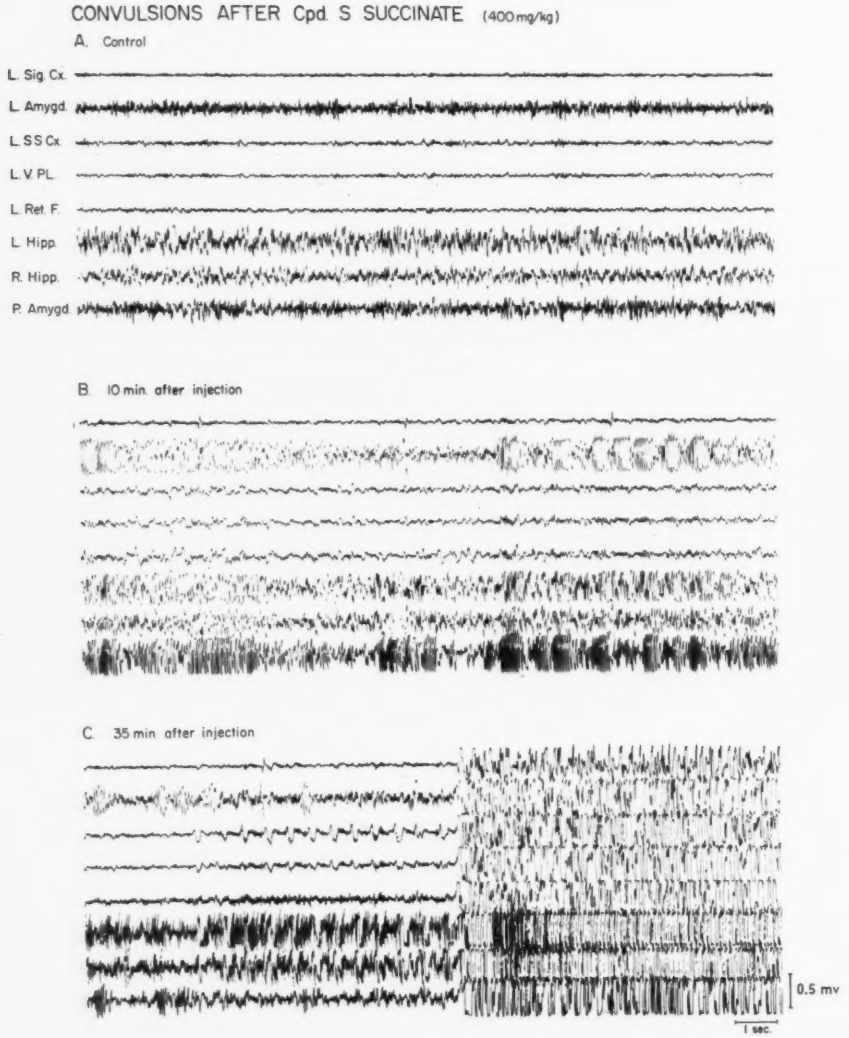

FIG. 2. A. Control record from chronically implanted, freely moving, cat. B. Rhythmic, high frequency amygdaloid activity. The cat shows all preconvulsive changes described in the text, but no overt seizures. C. Seizure activity begins in the dorsal hippocampal leads and becomes generalized. Abbr.: Sig. Cx. = Sigmoid (sensori-motor) cortex; SS Cx = Suprasylvian cortex; VPL = Thalamic n. ventralis postero-lateralis; Ret. F. = Reticular formation of the brain stem.

into paroxysmal spiking, developed in a fixed sequence in every case. It always started in both hippocampi (Fig. 2), followed a few seconds later by similar bursting in the amygdaloid nuclei (Fig. 3), then appeared in the hypothalamus, midbrain tegmentum and thalamus. This part of the sequence coincided in time with the first clinical signs: staring, salivation, mydriasis and the onset of tachypnea. Generalized high frequency "tonic" firing, followed by rhythmical after discharges in all leads accompanied the development of the grand mal convulsions. Varying degrees of depression of EEG activity ("isoelectric phase") followed each convulsion.

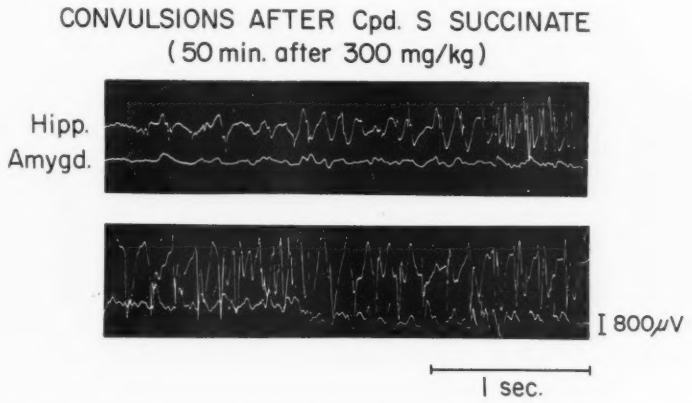

FIG. 3. Oscilloscopic registration on high speed moving film (acute experiment) shows that electrical seizure activity in the hippocampus precedes amygdaloid seizures.

The records were almost normal in appearance during the first few interictal periods. Later on, slow activity became progressively dominant as deterioration of the preparations set in.

Simultaneous oscilloscopic registration, on high-speed moving film, of records from electrodes placed above and below the pyramidal cell layer in the dorsal hippocampus showed clearly (Fig. 4) that convulsive firing in the layer of the apical dendrites preceded the appearance of similar activity in the records from the basal dendritic layer. The significance of this will be discussed elsewhere (Eidelberg, to be published).

Fig. 5 shows a record from an acute preparation 6 hours after intraperitoneal injection of Cpd. S in oil. At this time convulsive patterns followed each other within short intervals. It was interesting to note that, during the interval between seizures (Fig. 5), the hippocampus continued to fire while other leads showed typical flatness. Although this interictal firing of the hippocampus was clearly seen only in this particular cat, it underlines the prominent role of the hippocampus in these seizures.

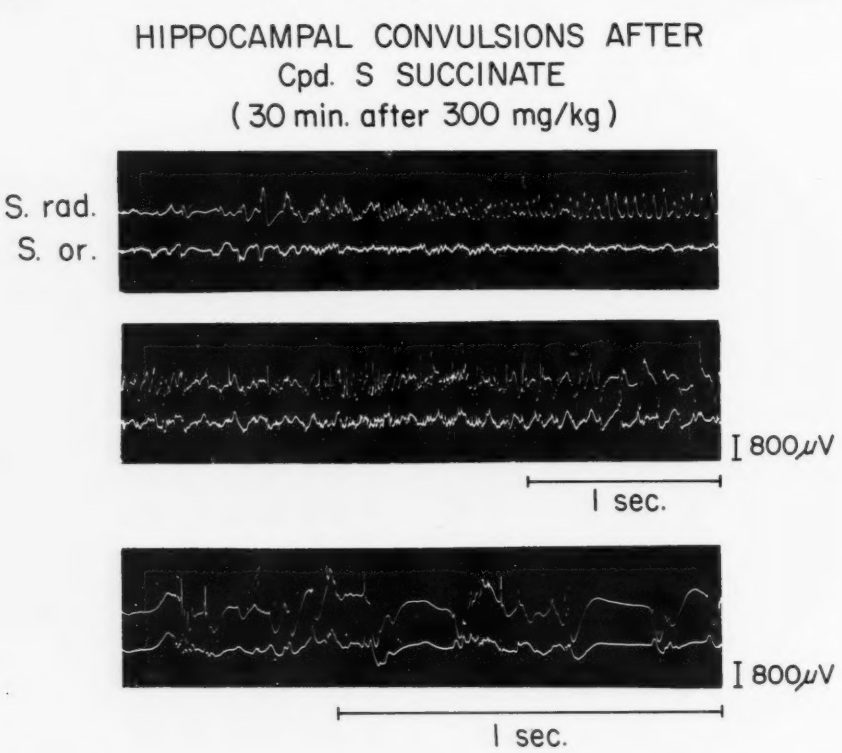

FIG. 4. Consecutive high-speed recordings (acute experiment). Electrical seizure activity first starts in the layer of the apical dendrites (=stratum radiatum) and shortly afterwards involves the layer of the basal dendrites (=stratum oriens) in the dorsal hippocampus.

The effect of drugs on convulsions in rats

While anesthetic drugs and steroids prevent or abolish steroid-induced convulsions (6, 7), none of the following substances had any significant long-lasting effect in this regard (groups of 6 rats each, including a control group): Chlorpromazine hydrochloride (5 mg./kg. i.p. 90 min. before injection of Cpd. S); Dibenamine (30 mg./kg. i.p. 90 min. before injection of Cpd. S); Hydroxylamine (60 mg./kg. i.v. 30 min. before injection of Cpd. S); Phenylisopropyl-hydrazine (Catron®) (10 mg./kg. i.p. 90 min. before injection of Cpd. S); Pyridoxine hydrochloride (up to 500 mg./kg. i.v. after first convulsion); Reserpine phosphate (5 mg./kg. i.p. 90 min. before injection of Cpd. S).

Diphenylhydantoin sodium was given intravenously in doses up to and exceeding 100 mg./kg. Only doses high enough to cause severe depression with loss of righting reflex diminished seizure activity. However, dilantin

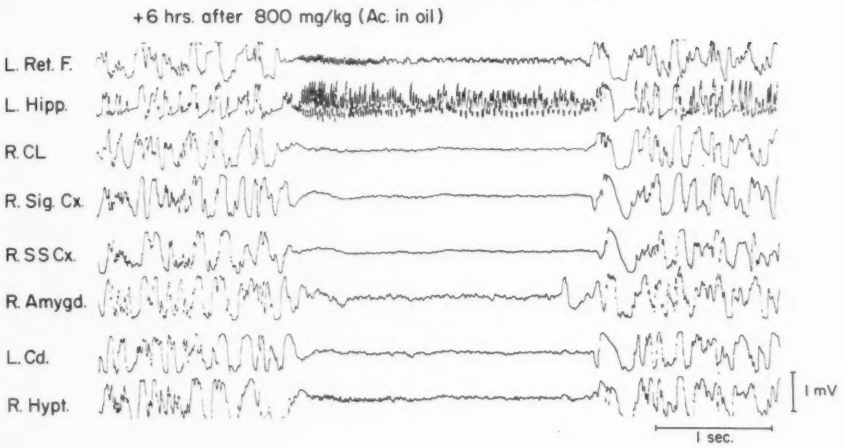

FIG. 5. Record during generalized convulsion following Cpd. S. It is seen that "tonic" discharges can be recorded from the hippocampus at a time when electrical activity is very much depressed in the rest of the brain.

never reduced the overall mortality. All these effects were observed regardless of whether dilantin was given after seizures had developed or up to 4 hours prior to seizure onset.

DISCUSSION

The results of these experiments clearly indicate that convulsive electrical activity can be recorded from limbic structures (hippocampus and amygdaloid nuclei) following administration of pharmacological doses of Reichstein's Cpd. S. This electrographic pattern is accompanied by a clinical picture essentially similar to that described many times before (10, 11, 12) when convulsions were induced by high frequency electrical stimulation or by local application of drugs or irritants to the hippocampus or amygdala. Generalization of this abnormal discharge to other brain structures was accompanied by clinical grand mal seizures.

While it is evident that the hippocampus and related structures are primarily involved in this convulsive activity, this should not be taken to imply that a selective effect of Cpd. S. on these structures is necessarily the cause. The reasons are essentially two: first, the hippocampus is known to have a very low threshold for generating convulsive discharges after mechanical or electrical stimulation (13), or in response to some substances not related to steroids, e.g. thiosemicarbazide (14), pyridoxine antimetabolites (15), insulin (16, 17), and even hypertonic saline (18). Secondly, a lowering of the threshold for the development of convulsions may have occurred in other structures to produce the full grand mal picture which followed the limbic and brainstem discharges. The general mecha-

nisms of hippocampal seizure propagation are very complex (19, 20, 21), and remain to be elucidated in detail.

Other important questions are related to the biochemical mechanisms involved in the induction of convulsions by steroid hormones.

The possibility that changes in brain gamma-aminobutyric (GABA) levels could be responsible for the development of these convulsions was ruled out by experiments on rats (unpublished). Direct measurements of GABA levels in various structures, including the hippocampus, did not indicate any significant changes. Interference with GABA levels by hydroxylamine and thiosemicarbazide did not change the pattern of the convulsions or the susceptibility of the animals.

During convulsions rats were hyperglycemic and showed no significant changes in their serum electrolytes (Na, K, Cl) (unpublished).

Tachypnea is a prominent feature of the clinical picture induced by the steroid in both species. The possibility that it could be causally (through respiratory alkalosis) involved in the convulsions was ruled out in the acute experiments in which the respiration of the animals was controlled externally.

The adrenals (22) and steroid hormones have been known for many years to influence greatly the function of the CNS. The work of Woodbury and associates (22) has shown that many steroids lower, while others increase, the electroshock seizure threshold when given on a pretreatment schedule. Many clinical reports point in the same direction. They indicate that sex hormones influence the time of onset and incidence of seizures in epileptic patients (23, 24, 25). Seizure discharges have also been observed in patients after treatment with corticoids (26). Whereas seizures after long exposure to relatively high levels of steroid hormones now constitute a well-known but still largely unexplained phenomenon, the fact that seizures can occur in mice (3, 4), and cats after a single injection of some steroids, is not so well known.

The possible difference in action after single, as opposed to multiple, administration of a hormone (or drug) should be stressed at this point. While Cpd. S, in small doses, chronically increases the electroshock seizure threshold (27), it has an apparently opposite effect when given acutely in high doses.—Cortisol is another case in point. Chronic treatment is known to decrease the electroshock seizure threshold markedly (27) while an acute overdosage (single injection up to 800 mg./kg.) induces neither convulsions nor anesthesia in rats (6, 7). However, the action of various anesthetics may be greatly potentiated by single injections of a high dose of cortisol (6, 7).—A single high dose of corticosterone induces anesthesia in rats (6, 7), but this compound has no comparable effect on the electroshock seizure threshold under chronic conditions (27).

It is well known, that after repeated administration, tolerance to various anesthetics (including certain steroids) develops so that their anesthetic

effects are diminished or disappear. This phenomenon may also play a role in explaining differences between acute and chronic effects of the same hormone (whether secreted internally or administered in higher doses).

With this in mind, a delicate balance of convulsant and anesthetic steroids can be envisaged. Its ultimate effect may depend not only on their blood or tissue level at any one moment but also on the preceding history of hormone output, and on additional conditioning factors.

One of the main objections to the assumption that Cpd. S or other steroids are in some way related to seizures in man is the fact that the dosage so far employed by us in cats and rats is very high, in the "pharmacological" range. However, man may be much more sensitive to these steroids than other mammals. It should also be pointed out that a dose sufficient to cause local seizure activity in the hippocampus is $\frac{1}{2}$ to $\frac{1}{4}$ of the dose which induces grand mal seizures. Also, minimal effective dose levels for any convulsive activity were not actually studied in our experiments, which were primarily aimed at first establishing the gross features of steroid-induced convulsions. Thus it may well be that in the future a steroid will be found which, at low dosages, may trigger seizures under more "physiological" conditions.

Whatever the actual role of Cpd. S or any other convulsive steroid in epilepsy or related conditions may be, we believe that steroid convulsions are of great interest. They provide a tool to induce and observe local (hippocampal and amygdaloid) and generalized intermittent convulsions for many hours in acute and chronic preparations. In their electrographic pattern and resistance to drug treatment they resemble temporal lobe epilepsy and may thus provide a model for therapeutically-directed research.

Addendum

Since this manuscript was submitted, preliminary experiments have shown that the Cebus monkey (Eidelberg, E., and C. A. Saldias: *J. Comp. Neurol.* **115:** 103, 1960) is much more sensitive than the cat or rat. Two monkeys had seizures within 10 min. and died 20–30 min. after an intraperitoneal injection of 160–200 mg./kg. of Cpd. S succinate. In rats and cats, this is not a fatal dose.—After 20 mg./kg. of this compound (i.p.), our first chronically implanted monkey showed intermittent periods of sleep with complete relaxation while in his chair. In the EEG record, these were accompanied by long-lasting "sleep-spindles." When more compound was injected, this pattern of intermittent "sleep" was even more pronounced until seizures eventually supervened.

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ACUTE REDUCTION IN EXTRACELLULAR FLUID VOLUME AND THE CONCENTRATION OF ANTIDIURETIC HORMONE IN BLOOD[1]

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ABSTRACT

A new chemical method for the extraction and purification of blood antidiuretic hormone (ADH) has been applied to the study of the effect of a reduction in extracellular fluid volume on the release of ADH in the dog. The extracellular fluid volume was reduced by peritoneal lavage with a hypertonic solution. This hypertonic solution had an electrolyte composition similar to that of interstitial fluid and a glucose concentration of 10%. Twenty minutes after the start of lavage the concentration of ADH in jugular vein blood had increased six-fold. At this time there was only a relatively small reduction in extracellular fluid volume. The plasma osmolality was increased by 4% as the result of an influx of glucose from the lavage solution. However, the increased blood concentration of ADH was apparently not the result of the increased plasma osmolality. When the plasma osmolality was elevated to a similar extent by the intravenous infusion of a concentrated glucose solution, the concentration of ADH in jugular vein blood was not affected. Thus, it is concluded that acute reduction in extracellular fluid volume results in increased release of ADH by the neurohypophyseal system.

IT IS widely held that the release of antidiuretic hormone (ADH) is influenced by changes in the volume of the interstitial compartment and/or the vascular compartment (see reviews by Smith (1) and Strauss (2). However, the evidence in support of this concept is largely indirect, based on experiments in which changes in urine volume in the experimental subject are taken as an index of changes in ADH release. Unfortunately, many of these experiments are further complicated by concomitant changes in solute excretion and renal hemodynamics. There have been no satisfactory studies in which the effect of changes in extracellular fluid volume on the blood level of ADH has been directly determined. The development by Sachs and his associates (3, 4) of a chemical procedure for the extraction, purification and concentration of small quantities of ADH in blood makes it possible to confer considerable specificity and sensitivity to the bioassay procedures. In the present investigation this method, modified for small

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volumes of blood and coupled with a sensitive antidiuretic bioassay, has been employed to determine ADH in blood under circumstances of a reduced extracellular fluid volume. Evidence is presented to support the hypothesis that a reduction in extracellular fluid volume results in an increased release of ADH.

METHODS

Extraction and purification of ADH. The method of Sachs and his associates (3, 4) has been modified for use with small volumes, 10–15 cc., of blood. The blood was added to 4 volumes of iced 12.5% trichloroacetic acid (TCA). The solution was stirred continuously with a magnetic stirrer during the addition of the blood. The mixture was then centrifuged briefly to separate the precipitated protein. The supernatant was saved and the precipitate extracted once with one volume of 10% TCA and twice with half volumes of 5% TCA. The supernatants were combined and filtered through Whatman, No. 12 filter paper. The TCA was removed from the supernatant by extraction 3 times with equal volumes of ethyl ether. The aqueous phase was brought to pH 4.5 with a dilute solution of NH_4OH. The extract was then passed through XE-64 resin, in the hydrogen form in a column one cm. in diameter and one cm. high at a flow rate of approximately one cc./min. The column was then washed with 10 cc. of water and the ADH was eluted with 25 cc. of a 30% pyridine—4% acetic acid solution. The eluate was collected in flasks containing 3 mg. of l-cystine in 0.5 ml. of 17.6% formic acid. The eluate was evaporated to dryness with a Rinco flash evaporator. The dried residue was taken up in several cc. of distilled water and evaporated to dryness. This process of addition of water and evaporation was repeated 5 more times to remove all traces of pyridine. The residue was taken up in a convenient volume of 0.9% NaCl—0.0285% acetic acid and centrifuged to remove the precipitated l-cystine. The solution was then ready for assay. The preparation of the XE-64 resin has been described by Weinstein *et al.* (3). The column chromatography was conducted at 4° C.

Bioassay of ADH. Male rats, weighing 300–400 gm. were deprived of food on the afternoon prior to the assay. On the morning of the assay, the rats were hydrated with warm tap water, 5 cc./100 gm. body weight, by gavage. Forty-five minutes later the rats were given 12% ethanol, 5 cc./100 gm. body weight, by gavage. A surgical level of anesthesia was usually achieved within 30 to 40 minutes. An external jugular vein was exposed and catheterized with polyethylene tubing (PE-20) for the injection of unknown and standard ADH solutions. The urinary bladder was exposed through a low, mid-line, abdominal incision. The urethra was ligated and the bladder catheterized with clear, flexible, plastic tubing (electrical insulating tubing) with an inside diameter of 0.066 in. This catheter led to two short lengths of stainless steel tubing separated by a short length of plastic tubing. The segments of stainless steel tubing served as electrodes. A final length of plastic tubing led to a graduated pipette so that the urine volume could be measured. Urine conductivity was recorded continuously by a modification of the method of Albers and Brightman (5). A small, constant current was passed between the two electrodes in the bladder catheter and the voltage drop between these electrodes was recorded on a Wheelco multipoint recorder.[2] The administration of ADH to the test animal results in an antidiuresis which is reflected by an increased conductivity of the urine and a decreased voltage drop between the electrodes. The area under the conductivity curve was taken as the index of response. In our hands this has proven to be a more

[2] A detailed description of the apparatus used for recording the conductivity of the urine will be supplied upon request.

uniform index of response than some function of change in urine volume. The stomach was catheterized via the esophogus with polyethylene (PE-90) tubing. During the assay the levels of hydration and anesthesia were maintained by replacing each cc. of urine with one cc. of 2% ethanol given via the stomach tube. The sensitivity of the rat was tested by the initial injection of 80 μU. of standard pitressin. This response was not included in the assay. The U.S.P. posterior pituitary reference standard, with a potency of 2 U./mg. was used as the standard in the bioassay. A 2×2 factorial design was used to estimate the potency of the unknown solutions. A complete assay consisted of 4 injections of standard and 4 injections of unknown, each at 2 dose levels, in the same rat. Because of limitations of sample size, it was not possible to perform complete assays on many samples. However, no assay was included unless it was based on at least 2 injections of standard and 2 injections of unknown, all in the same rat. The log dose response curve was rectilinear between 20 and 100 μU. The lambda for this assay, calculated from data obtained in 13 dose-response curves, was 0.135. For each dose-response curve, standard vasopressin was injected at 4 dose levels. Each dose level was given in duplicate, and the order of injection was randomized.

Experimental procedure. Male dogs were anesthetized with sodium pentobarbital, 30 mg./kg. body weight. Mean arterial blood pressure was recorded with a mercury manometer from a cannula in a femoral artery. Samples of blood, each 20 to 21 cc. in volume, were collected in heparinized syringes from a cannula directed toward the head in an external jugular vein. Fifteen cc. of blood were used for analysis of ADH. The remainder of the blood was used for the estimation of the hematocrit, the plasma concentrations of sodium and potassium and the plasma osmolality. Immediately prior to taking the blood sample, 10 cc. of blood were withdrawn to "wash out" the dead space of the cannula and the length of jugular vein leading to the head. This blood was returned to the dog via a cannula in a femoral vein. The lavage procedure was begun 3 to 5 minutes after taking an initial blood sample. The lavage solution had an electrolyte composition similar to that of interstitial fluid (6) and was made hypertonic with glucose (10%). The lavage solution was given intraperitoneally, 50 cc./kg. body weight, over 3 to 4 minutes and remained in the peritoneal cavity for the duration of the experiment. Additional blood samples were taken 20, 40 and 60 minutes after the start of lavage. In a control series of experiments the same procedure was followed, except that the concentration of glucose in the lavage solution was 0.1%. Sodium and potassium analyses were performed by internal standard flame photometry and total solute concentrations were estimated with a Fiske osmometer.

RESULTS

Recovery of ADH from dog blood. The results of recovery experiments in which known quantities of U.S.P. posterior pituitary reference standard were added to dog blood are summarized in Table 1. Recoveries averaged approximately 40% and were independent of the concentration of ADH

TABLE 1. RECOVERY OF ADH FROM 10 CC. VOLUMES OF DOG BLOOD

Added mU.	No. of samples	% recovery
0.5	7	41.4 ± 2.6*
1.0	14	42.7 ± 2.2*
2.0	2	41.2, 36.8

* Means ± S.E.

over the range studied, 0.5 to 2 mU. per 10 cc. blood. This range was selected because it coincided with the amounts of ADH usually found in blood during the lavage procedure. Weinstein *et al.* (3), using essentially the same method for isolation, but using the pressor assay, reported an average recovery of 60% when 50 to 400 mU. of ADH were added to 100 cc. volumes of dog blood.

TABLE 2. EFFECT OF PERITONEAL LAVAGE ON CONCENTRATION OF ADH IN BLOOD DRAINING FROM THE HEAD

	Pre-lavage	Min. after start lavage		
		20	40	60
	A. 10% glucose in lavage soln.			
ADH* μU/cc.	3.9 ± 1.0 (9)	22.8 ±4.7 (9)	46.4 ±7.6 (9)	62.3 ±11.0 (9)
Hct %	39.9 ± 0.9 (9)	43.8 ±1.0 (9)	47.5 ±0.9 (9)	50.3 ± 0.8 (9)
MABP mm. Hg	140 ± 6 (9)	146 ±4 (9)	142 ±4 (9)	127 ± 5 (9)
Plasma Os. mOs./kg	299 ± 4 (3)	312 ±4 (3)	318 ±7 (3)	329 ± 4 (3)
Plasma Na m.eq./l.	151 ± 2 (4)	150 ±2 (4)	152 ±2 (4)	151 ± 2 (4)
Plasma K m.eq./l.	3.68 ± 0.11 (4)	3.40 ±0.12 (4)	3.23 ±0.33 (4)	3.30 ± 0.36 (4
	B. 0.1% glucose in lavage soln.			
ADH* μU/cc.	3.8 ± 1.6 (6)	3.2 ±1.4 (8)	3.2 ±0.9 (8)	6.6 ± 2.3 (7)
Hct %	41.1 ± 1.5 (8)	41.9 ±1.7 (8)	42.9 ±1.8 (8)	42.4 ± 1.8 (8)
MABP mm. Hg.	136 ±11 (8)	143 ±7 (8)	144 ±6 (8)	143 ± 6 (8)
Plasma Os. mOs./mg.	309 ± 1 (4)	308 ±1 (4)	309 ±2 (4)	307 ± 3 (4)
Plasma Na m.eq./l.	149 ± 1 (4)	147 ±1 (4)	145 ±1 (4)	148 ± 1 (4)
Plasma K m.eq./l.	3.71 ± 0.17 (4)	3.63 ±0.17 (4)	3.66 ±0.16 (4)	3.67 ± 0.15 (4)

Os. = osmolality; Hct = hematocrit; MABP = mean arterial blood pressure.
Means ±S.E.; number in parentheses = number of experiments.
* Not corrected for % recovery.

Reduction of extracellular fluid volume by peritoneal lavage. Extracellular fluid volume was reduced by peritoneal lavage with a solution which had an electrolyte composition similar to interstitial fluid (6) and which was made hypertonic by the addition of glucose (final glucose concentration was 10%). This procedure resulted in a six-fold increase in the concentration of ADH in jugular vein blood within 20 minutes after the start of lavage (Table 2 A). An approximation of the change in extracellular fluid volume

was obtained from the change in hematocrit,[3] assuming that there were no changes in the volume or number of the red blood cells and that the percentage change in plasma volume equalled the percentage change in extracellular fluid volume. On this basis, the extracellular fluid volume was reduced by 15% 20 minutes after the start of lavage. During the next 40 minutes of lavage, the concentration of ADH in jugular vein blood progressively increased as the extracellular fliud volume decreased. The mean arterial blood pressure remained relatively constant until near the end of the experiment. Sixty minutes after the start of lavage it had dropped about 11% from the value obtained at 40 minutes. The lavage procedure was without effect on the plasma concentration of sodium. The plasma concentration of potassium dropped slightly during the lavage procedure but these changes were not statistically significant. In each of the 3 experiments in which measurements were made, the plasma osmolality increased during lavage, apparently as the result of the influx of glucose from the lavage solution. The average increase in plasma osmolality after 20 minutes of lavage was 4%. In a control series of experiments in which an isotonic lavage solution was used (the glucose concentration was 0.1%), there were no significant effects on the concentration of ADH in jugular vein blood nor on any of the other parameters measured (Table 2 B).

Elevation of plasma osmolality by the infusion of hypertonic glucose solution. At this point it was not certain whether the increased concentration of ADH in jugular vein blood was the result of a reduction in extracellular fluid volume or the elevation of the plasma osmolality. Consequently, another series of experiments was performed in which an attempt was made to increase only the osmolality of the plasma by the infusion of a concentrated glucose solution. A solution which contained glucose at a concentration of 36% and which had the same electrolyte composition as the lavage solution was infused intravenously at a rate which varied between 2.0 and 2.6 cc./min. Blood samples were taken from the cannulated, cephalad end of an external jugular vein just prior to and at 20 minutes and 40 minutes after the start of the infusion. The data from these experiments are summarized in Table 3. Twenty minutes after the start of the infusion, the plasma osmolality was elevated to a level comparable to that found 20 minutes after the start of lavage in the "lavage" experiments. There was, however, no increase in the concentration of ADH in jugular vein blood. There was an increase in the blood level of ADH 40 minutes after the start of the in-

[3] The percentage change in plasma volume was calculated as follows: let het_1 = the initial hematocrit and X_1 = the initial plasma volume; the subscript 2 will denote these same functions at a later time.

$$\frac{X_2}{X_1} = \left[\frac{1 - het_2}{1 - het_1} \cdot \frac{het_1}{het_2}\right] 100$$

fusion, coincident with a further rise in the plasma osmolality. The magnitude of this increase in the blood level of ADH was less than that found in the lavage experiments. These infusion experiments were unfortunately complicated by a fall in the hematocrit. This indicates that there was an increase in the vascular volume and probably also an increase in interstitial fluid volume, due to a movement of water out of cells as a result of the increased osmotic pressure of the extracellular fluid.

Since in these "infusion" experiments an increase in the osmotic pressure of the plama comparable to that observed 20 minutes after the start of lavage in the "lavage" experiments was without effect on the blood level of ADH, it would appear that the increase in the blood level of ADH found 20

TABLE 3. EFFECT OF ELEVATION OF PLASMA OSMOLALITY BY THE INFUSION OF HYPERTONIC GLUCOSE SOLUTION ON CONCENTRATION OF ADH IN BLOOD DRAINING FROM THE HEAD

	Pre-infusion	Min. after start	
		20	40
ADH* μU/cc.	4.3 ±1.4 (5)	2.7 ±1.6 (5)	11.1 ±2.8 (4)
Hct %	42.9 ±1.9 (5)	37.7 ±1.5 (5)	37.4 ±2.2 (5)
MABP mm. Hg	138 ±2.8 (5)	149 ±4.5 (4)	144 ±5.6 (5)
Plasma Os. mOs./kg.	301 ±1 (5)	314 ±2 (5)	321 ±4 (5)
Plasma Na m.eq./l.	140 ±2 (4)	137 ±3 (4)	139 ±3 (4)
Plasma K m.eq./l.	3.50 ±0.15 (4)	3.33 ±0.28 (4)	2.97 ±0.11 (4)

Means ±S.E.; number in parentheses = number of experiments.
See Table 2 for abbreviations.
* Not corrected for % recovery.

minutes after the start of lavage was the result primarily of the reduction in extracellular fluid volume. It could be argued that an increase in the concentration of ADH in jugular vein blood was not obtained in the "infusion" experiments because the effect of the elevated plasma osmolality was blocked by the increased extracellular fluid volume. However, such an argument itself implies that the release of ADH is influenced by changes in extracellular fluid volume. Furthermore, Verney (7) found that glucose was less effective than equiosmolar quantities of sodium salts or sucrose in inhibiting a water diuresis in dogs.

During the course of the infusion of the hypertonic glucose a progressive fall in the plasma concentration of potassium was observed, although the plasma concentration of sodium remained constant. This phenomenon has been observed previously (8).

DISCUSSION

The evidence in support of the concept that ADH release is influenced by changes in extracellular fluid volume has been largely derived from experiments in which the blood titer of ADH was not assayed. There have been

only a few attempts to determine the effect of changes in extracellular fluid volume on the blood level of ADH. Leaf and Mamby (9) studied the effect of the administration of 5% glucose into the peritoneal cavity of dogs. This procedure depleted the extracellular fluid of electrolyte and, presumably, also reduced the extracellular fluid volume, although no data are given for this latter parameter. These authors reported an impaired ability to excrete a water load and an increased concentration of antidiuretic material in the plasma. Ginsburg (10) and Weinstein, Berne and Sachs (3) found that hemorrhage resulted in an increased release of ADH, but these experiments were complicated by changes in blood pressure. On the other hand, Baratz and Ingraham (11) were unable to detect a change in the concentration of ADH in plasma when the vascular volume was expanded 25% by infusing intravenously 6% dextran in isotonic saline solution.

With the exception of the work of Weinstein, Berne and Sachs (3) the methods used in these experiments for the measurement of ADH in plasma are open to criticism. First, the antidiuretic assay is not specific for ADH. Urine volume can be greatly altered by changes in glomerular filtration rate or solute excretion. Furthermore, Berliner and Davidson (12) have shown that dogs with diabetes insipidus excrete a hypertonic urine when the glomerular filtration rate is reduced. Consequently, the administration of whole plasma to the assay animal as was done in the work of Leaf and Mamby (9), Ginsburg (10) and Baratz and Ingraham (11) may give misleading results, since plasma may contain substances other than ADH which will inhibit a water diuresis and induce the formation of a concentrated urine. Thus, Heller (13) has reported that plasma from animals subjected to ether anesthesia, when injected into hydrated, ethanol-anesthetized rats, results in an antidiuresis and a reduction in-creatinine excretion. Heller considered the decreased excretion of creatinine to indicate a reduction in glomerular filtration rate, and he suggested that the reduction in glomerular filtration rate contributed to the antidiuresis. Second, when plasma is assayed directly, the sensitivity of the bioassay is limited by the amount of plasma which can be injected into the assay animal, usually the rat. Third, the bioassay used by Leaf and Mamby (9) is open to question since the test substances were administered intraperitoneally. This procedure has been shown by Ames and van Dyke (14) to give aberrant results. These problems have been largely circumvented in the present experiments. The chemical method devised by Sachs and his associates (3, 4) for the extraction and purification of ADH in blood confers considerable specificity upon the bioassay procedure. This method also greatly increases the sensitivity of the bioassay by making it possible to concentrate into a small volume the ADH contained in a given volume of blood. An additional test of this procedure was made. The antidiuretic material obtained in one of the "lavage" experiments was inactivated by treatment with trypsin, which has been shown to inactivate ADH, but not oxytocin (15).

In the present experiments direct confirmation has been provided for the

concept that changes in extracellular fluid volume influence the release of ADH from the neurohypophyseal system. Reduction of the extracellular fluid volume resulted in a marked increase in the concentration of ADH in the blood draining from the head before there was any detectable change in systemic mean arterial blood pressure. These experiments were complicated to some extent by a rise in the osmotic pressure of the plasma, due to the influx of glucose from the lavage fluid, concomitant with the rise in the blood titer of ADH. However, it was shown in a separate series of experiments that a comparable elevation of the plasma osmolality by the infusion of glucose was without effect on the concentration of ADH in jugular vein blood. We have considered the increased concentration of ADH in jugular-vein blood to reflect an increased release of ADH by the neurohypophyseal system. However, an increased concentration of ADH would also be observed in blood draining from the head under circumstances in which the rate of secretion of ADH remains constant but the cerebral blood flow falls. A six-fold increase in the concentration of ADH in jugular vein blood was observed 20 minutes after the start of peritoneal lavage, when the extracellular fluid volume was reduced by roughly 15% and the mean arterial blood pressure was unchanged. It would appear to be highly unlikely that under these conditions the cerebral blood flow was reduced to one-sixth of its pre-lavage value. On the basis of these various considerations it is concluded that a reduction in extracellular fluid volume, independent of changes in systemic mean arterial blood pressure and plasma osmotic pressure, results in an increased release of ADH.

The use of changes in hematocrit to estimate changes in extracellular fluid volume was based on three assumptions, *i.e.*, that there were no changes in the number or volume of the red blood cells and that changes in plasma volume were accompanied by proportional changes in extracellular fluid volume. The first assumption is probably valid in view of the short duration of the experiments and the small volume of the blood samples. The second assumption is not completely valid, because the increased osmotic pressure of the plasma during lavage would probably have resulted in a reduction in the volume of the red blood cells. Thus the change in hematocrit would tend to underestimate the reduction in plasma volume and extracellular fluid volume. However, the error due to this factor is quite small 20 minutes after the start of lavage, since the osmolality of the plasma was elevated only 4% at this time. The third assumption is apparently valid. Elkinton *et al.* (16) have shown that in acute water depletion plasma volume diminished in proportion to the fall in extracellular fluid volume. In order to obtain a further check on the effectiveness of the peritoneal lavage, the lavage fluid was drained from the peritoneal cavity at the conclusion of each experiment, 60 minutes after the start of lavage. In the control experiments with isotonic lavage fluid, an average of 80.4% of the administered fluid was recovered. This figure was used to correct the vol-

ume of fluid recovered in the hypertonic lavage experiments. On this basis, an average of 744 cc. of fluid were recovered in excess of the amount originally placed in the peritoneal cavity in the hypertonic lavage experiments. The reduction in extracellular fluid volume calculated from the change in hematocrit and the assumption that the extracellular fluid volume is equivalent to 20% of the body weight (an average of 15.5 kg.) was 1066 cc. The reason for this discrepancy is not apparent, but it would appear that the change in hematocrit as we have used it overestimates the change in extracellular fluid volume. However, the important point is that 20 minutes after the start of hypertonic lavage, the time when the biggest increase in the blood titer of ADH was observed, there was a small reduction in extracellular fluid volume. A reasonable estimate of this reduction is 15%, calculated from the change in hematocrit, although the actual value probably lies between 10 and 20%.

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EFFECT OF RESERPINE AND CATECHOLAMINES ON JUXTAGLOMERULAR CELLS IN RATS FED NORMAL AND LOW SODIUM DIETS[1]

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ABSTRACT

Augmentation of the granular cell index in the rat juxtaglomerular apparatus by either short or prolonged dietary salt restriction was prevented by one injection of reserpine or by four injections of norepinephrine administered at the onset of salt restriction.

Four subcutaneous injections of norepinephrine reduced low salt elevated GCI when given after twenty-nine days of low salt intake but failed to reduce the elevated granular cell index of adrenalectomized, cortisone or salt supported rats.

These results are consistent with the hypothesis that the granularity of juxtaglomerular cells is inversely related to the mineralo-corticoid level.

DUNIHUE and Robertson (1) and Wiedman, Dunihue and Robertson (2), have shown that the incidence of granular cells in the juxtaglomerular apparatus (JGA), expressed as the granular cell index (GCI), is inversely related to the mineralo-corticoid level (MC) but that reduction of GCI by a typical mineralo-corticoid, desoxycorticosterone, occurs only when sodium intake is adequate. The Hartrofts (3) first reported and we confirmed (2) an apparent deviation from this inverse relationship of GCI to MC level in normal rats fed low salt diet. In these animals the GCI was increased despite high endogenous MC. We suggested that this apparent exception could be explained by the assumption that low salt stress caused an early, transient reduction of steroid output during which GCI rose. Once elevated the index could not be reduced by the subsequently high endogenous MC because of inadequate sodium intake. Support for this assumption was offered by our finding (2) that daily supplements of exogenous MC, given in decreasing amounts during only the first half of a low salt regime, prevented increase of GCI.

The observations of Knigge *et al.* (4) that reserpine blocks the early, temporary reduction of steroid output in heat stressed rats suggest to us

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that this drug might also block the transient decrease of corticoids in low salt stressed rats, thereby preventing an increase in GCI. Since reserpine causes release of epinephrine and norepinephrine, we have investigated the action of these catecholamines as well as reserpine on the GCI elevating effect of low salt intake.

MATERIAL AND METHODS

Wistar strain, male rats weighing 200 gm., were fed pelleted, low sodium diet containing 77% powdered food similar to whole milk (Lonalac, Meade Johnson Co.), 12% sucrose, 7.8% finely ground cellulose (Alphacel, Nutritional Biochemical Co.), 2% vitamin diet fortification mixture (NBC), and 1.2% of a mixture of known essential minerals except sodium. This diet contained less than 0.02% sodium; a diet with normal sodium content was secured by adding 0.7% NaCl to the above mixture and reducing Alphacel by the same amount.

Reserpine, 2.5 mg./ml. in 5% dextrose was injected subcutaneously at a dosage level of 50 μg/100 gm. of body weight. Norepinephrine in 5% dextrose and epinephrine in 0.9% saline, 200 μg/ml., were injected subcutaneously at the level of 80 μg/100 gm. of body weight. Five percent dextrose in equivalent volumes was used for control injections.

Adrenalectomies, in experiment 4, were performed in one stage. GCI were determined on Zenker-formol fixed, Masson stained sections by counting all glomeruli in one or more sections of each kidney and recording the number of times granular cells, singly or in groups, were encountered per 100 glomeruli.

Experimental Design

Two experiments were designed to determine the effect on GCI of reserpine and catecholamines administered during the early stages of low salt stress when we assume adrenal steroid output may temporarily decrease.

Experiment 1. Thirty rats were fed the normal and another 30 the low salt diet for 4 days. One-third of the rats on each dietary regime were injected with a single dose of reserpine 24 hours after being placed on the diet; another one-third received 4 injections of norepinephrine between the 22nd and 42nd hours after the beginning of the dietary period; and one-third were injected with an equivalent volume of dextrose on the same schedule used for norepinephrine.

Experiment 2. The plan for this experiment was identical to that of Experiment 1 except that the ditary period was extended to 29 days and a fourth group of 10 rats was added to each dietary regime. These additional groups were injected with epinephrine on the same schedule used for norepinephrine.

Another experiment was designed to determine the effect of reserpine and catecholamines on GCI elevated by approximately one month of low salt stress and at a time when adrenal minerocorticoid output is known to be increased (5).

Experiment 3. Twenty-one rats were fed the low salt diet. After 29 days on this regime, 11 rats were given 4 injections of norepinephrine over a 20-hour period and 10 received dextrose injections on the same schedule. The rats were killed 4 hours after the last injection.

A fourth experiment was designed to ascertain the effect of norepinephrine on GCI elevated by MC deficiency.

Experiment 4. Forty rats were adrenalectomized, placed on normal diet and divided into two equal groups. One group was supported by the addition of 1% NaCl to the drinking

TABLE 1. BLOCKING EFFECT OF RESERPINE AND CATECHOLAMINES ON ELEVATION OF GRANULAR CELL INDEX BY LOW NaCl INTAKE

Treatment	Dietary period			
	Experiment 1, 4 days		Experiment 2, 29 days	
	NaCl Intake		NaCl Intake	
	Normal	Low	Normal	Low
	GCI ±S.E.	GCI ±S.E.	GCI ±S.E.	GCI ±S.E.
Dextrose	11.3±1 (10)	16.9±1.2* (10)	12 ±1.5 (9)	17.5±1* (10)
Reserpine	10.1±1.2 (10)	12.3±1 (9)	12.2±1.3 (10)	15.6±1.4 (8)
Norepinephrine	10.4±1.3 (9)	12.4±1.1 (10)	11.7±1.1 (10)	10.7±1 (10)
Epinephrine			13.1±2 (6)	14.9±1.8 (10)

* Significant elevation of GCI $P < 0.05$.
Number of surviving animals in each group is given in parentheses.

water, the other by daily injections of 0.5 mg. of cortisone. Twenty-nine days after adrenalectomy, half of the animals in each group were given 4 injections of norepinephrine over a period of 20 hours; the others received corresponding injections of dextrose. All rats were killed 4 hours after the last injection.

RESULTS

As shown in Table 1, reserpine and norepinephrine blocked or prevented augmentation of GCI induced by feeding a low salt diet for 4 days. In a longer period of low salt stress, norepinephrine blocked completely the elevation of GCI, while reserpine and epinephrine showed effects intermediate between that of dextrose (no blocking) and norepinephrine (complete blocking). In the dosages used, neither reserpine nor the catecholamines affected the GCI of rats fed normal sodium diets.

The data in Table 2 show that norepinephrine, injected after 29 days of a low salt regime, reduced the elevated GCI produced by this period of low salt intake, even though no additional sodium was given. Norepinephrine,

TABLE 2. EFFECT OF NOREPINEPHRINE ON GRANULAR CELL INDEX ELEVATED BY LOW SALT INTAKE AND BY MINERALO-CORTICOID DEFICIENCY

Experimental group	Treatment	
	Dextrose	Norepinephrine
	GCI ±S.E.	GCI ±S.E.
29 days 3. Low Salt Intake	17.5±1 (10)	11.3±1.3* (11)
29 Days 4. Adrenx Salt Support	20.2±2.4 (10)	22.1±1.3 (10)
29 Days 4. Adrenx Cortisone Support	19.5±1.8 (10)	18.4±1.4 (7)

* Significant reduction of GCI $P < 0.05$.
Number of surviving animals in each group is given in parentheses.

however, failed to reduce GCI elevated by 29 days of MC deficiency in either salt or cortisone supported, adrenalectomized rats.

DISCUSSION

The results show unequivocally that norepinephrine and to a lesser extent reserpine and epinephrine prevent GCI augmentation by low salt intake. To this extent the data support the suggestion that reserpine, or products released by it, might block elevation of the GCI by preventing a transient decrease of corticoids in the early stages of low salt stress. While there is no direct evidence that reserpine and catecholamines prevent an increased GCI in low salt stressed rats by maintaining MC secretion, these compounds are known to stimulate adrenal secretion (6, 7). Further, reserpine has been shown to prevent a temporary decrease of steroid output in heat stressed rats (4).

The ability of norepinephrine to reduce low salt elevated GCI in non-adrenalectomized rats (high MC) in contrast with its inability to lower the elevated GCI in adrenalectomized rats (MC deficient), even in the presence of added salt or glucocorticoids, reemphasizes the essential role of mineralo-corticoids in GCI regulation.

Since the results show that norepinephrine can reduce low salt elevated GCI, an adequate sodium intake is not, as previously thought (2, 3), a prerequisite for lowering of the GCI. This does not, however, preclude a role for sodium in GCI reduction, for, as Friedman has suggested (8), the extra-intracellular sodium gradient (9) of the JGA cells may be more important than sodium intake *per se*. This gradient is probably higher than normal in low salt fed rats in view of the decreased intracellular smooth muscle sodium in such animals (10). Norepinephrine conceivably could reduce this gradient, since it is known to cause a loss of potassium and a gain in smooth muscle sodium (10, 11, 12). Therefore, as a speculation, we suggest that norepinephrine reduces low salt elevated GCI by shifting the extra-intracellular sodium gradient of the JGA cells towards normal, thereby permitting the high MC level prevailing during salt restriction to reduce the GCI. It appears, however, that this gradient could play only a permissive role in GCI reduction, since norepinephrine is unable to decrease the GCI in the absence of mineralo-corticoids (Experiment 4).

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EFFECTS OF THYROTROPIN ON THYROIDAL WATER AND ELECTROLYTES IN THE CHICK[1]

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ABSTRACT

Some aspects of electrolyte and water metabolism of thyroid tissue have been studied in the 1½-day-old chick, with and without thyrotropin. In the untreated chick, each Kg. of thyroid tissue contained 723 gm. of water, 111 meq. of sodium and 60 meq. of potassium. The volume of distribution of radiosodium in the thyroid expanded progressively over a period of 24 hours, ultimately reaching approximately 60 percent of organ weight. By contrast, in nine extrathyroidal tissues studied, equilibration with radiosodium was very rapid. Thirty minutes after the intracardiac administration of TSH, the thyroid gland showed a slight increase in water concentration, a marked increase in the 10-minute uptake of radiosodium, no significant change in the concentration of stable sodium or potassium, and no change in the 10-minute volume of distribution of radio-iodinated human serum albumin. Four hours after TSH, there was a more striking increase in water concentration, no effect on radiosodium uptake, a decrease in the concentration of stable sodium, and an increase in the concentration of stable potassium. The latency of the effect of TSH upon thyroidal radiosodium uptake was 15 minutes or less. The threshold dose was 0.49 milliunits (U.S.P.) per chick.

The data indicate the presence in the thyroid of an "extra" extracellular space, the follicular lumen, which is rich in sodium and in which exchange with labelled sodium is slowly accomplished. The action of TSH in the first half-hour is interpreted as an acceleration of the transfer of sodium across one or more thyroidal barriers. The effects of TSH observed after four hours are indicative of cellular hypertrophy and loss of intraluminal colloid.

THE concept has re-emerged in recent years that hormones may function by altering the permeability of a target tissue to various substances (1–3). In the case of the uterus, several early actions of estrogen suggest the presence of such a mechanism; these include the induction of hyperemia, an increase in water content, and increases in the tissue uptake of trypan blue, Na^{24}, P^{32} and radio-iodinated albumin (3–7). Similarly, testosterone administration results in an increase in the water content of the seminal vesicle of the castrate rat (8). Recently, Eichhorn *et al.* demonstrated that the administration of ACTH leads rapidly to a significant expansion of the intraadrenal volume of distribution of D-xylose in hypophysectomized rats (9), an action not unlike that of insulin on skeletal muscle (10).

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Two observations suggesting a similar action of thyrotropin (TSH) upon the thyroid gland have been reported. *In vitro*, TSH causes a relative increase in the water content of beef thyroid slices. However, this effect is slow in development; it becomes evident only after 10 hours and is still increasing after 25 hours (11). In the rat, TSH causes a decline in the thyroid-to-serum concentration gradient of iodide, before it induces its more characteristic action of increasing that gradient (12). Analysis of these data indicated that an enhanced efflux of iodide was the immediate mechanism for the transient decline, and Halmi *et al.* proposed an alteration in the permeability of thyroidal cell membranes as a possible underlying mechanism (12). In the present study, the early effects of TSH *in vivo* upon the content of water, sodium and potassium and upon the uptake of radioactive sodium of the chick thyroid gland were explored, in order to determine whether stimulation of the thyroid gland is accompanied at an early stage by altered rates of transfer of these inorganic constituents. A preliminary communication of part of this work has been published (13).

METHODS

White Leghorn cockerels, supplied by Kimber Farms, Pomona, Calif., were studied and sacrificed during the second day after hatching, unless otherwise indicated. During acute experiments, chicks were kept at room temperature without food and water; when kept for longer periods, as in the experiments of Figure 1 and in experiment 12 (Table 2), they were warmed by a heat-lamp and given access to water.

Hormonal agents were dissolved in isotonic saline immediately before use and injected by the intracardiac route in a volume of 0.1 ml. The dose of bovine TSH ordinarily employed was 10 milliunits (U.S.P.) and the preparation, unless otherwise indicated, was Thytropar®. Control animals received 0.1 ml. of isotonic saline by the same route.

Isotopic materials in isotonic saline were injected in 0.1 ml. of volume into the right jugular vein. Amounts employed were 10 μc of Na^{24}, 4 μc of Na^{22} or 10 μc of radio-iodinated human serum albumin (RISA). Before use, $Na^{24}Cl$ in HCl (Oak Ridge National Laboratory) was neutralized with 0.2 M NaOH and diluted to a final concentration of 300–350 milliosmoles per liter. Radio-iodinated albumin in saline was dialyzed before use against 10 L. of isotonic saline, to which sufficient unlabelled human serum albumin was added to make it isosmotic with the contents of the bag (0.16% albumin). The dialysis was carried out at 4° C for 4 hours, and reduced the TCA-soluble fraction to less than 0.5% of the total radioactivity. The electrophoretic mobility of dialyzed RISA was identical to that of human serum albumin, as determined by the staining and counting of strips containing RISA alone and of strips containing RISA plus added unlabelled albumin.

Chicks were sacrificed by decapitation, blood was collected by gravity into heparinized tubes, and the thyroid glands and other tissues were dissected quickly, trimmed on filter paper moistened with saline, blotted lightly, placed in tared shell vials and weighed. Vials were dried overnight at 105° C, weighed and counted in a well-type scintillation counter against an appropriate aliquot of the administered Na^{24} or Na^{22}. In the various experiments on thyroidal water content, the thyroid glands of two, three, six or ten animals were pooled in glass-stoppered weighing vials. Within a given experiment, the same number of thyroids was employed per vial. In the studies of stable sodium and potassium content, the procedures were as above except that tissues were trimmed on filter paper moistened with distilled water. After determination of dry weight, the tissues were trans-

ferred to small beakers with 2.0 ml. of concentrated nitric acid and digested overnight at room temperature and then to dryness at 80° C. The digests were dissolved in warm water, filtered through #40 Whatman paper, diluted to 25 ml. and analyzed by flame photometry. The electrolyte content of a reagent blank was simultaneously determined and was subtracted from that of the tissues. The recovery of sodium added to pools of chick thyroids averaged 102%. The electrolyte content of serum or of heparinized plasma was determined after dilution of the samples, without digestion. The amount of heparin employed (2 mg.) introduced approximately one milliequivalent of sodium per liter of plasma.

The concentration of sodium or of radiosodium was calculated as the content in the organ per gram of tissue weight or per gram of tissue water. The tissue concentration divided by that for plasma describes the apparent volume of distribution of sodium or of radiosodium as a fraction of the total weight of tissue or of tissue water. Corrections for the Donnan equilibrium and for the difference between the water content of extracellular fluid and of plasma were not introduced, since they essentially cancel each other. The "true" volumes of distribution would actually be the volume herein reported, multiplied by $0.942^2/0.95$, or 0.992.

In order to check on the reliability of blood samples obtained by decapitation, the hematocrit of 5 such samples was compared with the hematocrit of samples drawn by syringe from the heart immediately prior to decapitation. The hematocrits resulting from the two methods were essentially identical. However, in those experiments in which the chicks were allowed food and water, the crop was clamped prior to decapitation as an added precaution against dilution of blood by extravascular materials.

RESULTS

Thyroidal Weight and Water Content. The over-all mean thyroidal water concentration in pools representing a total of 316 saline-treated control chicks was 72.3 mg. per 100 mg. of thyroid weight during the second day of life. The thyroidal water concentration was lower than that of muscle, testis, duodenum, kidney, glandular stomach, thymus and adrenal; it exceeded the water concentration only of organs rich in lipid—liver and subcutaneous fat. There was considerable inter-experiment and inter-group variation in the water content of the thyroid of control animals. In order to minimize the importance of this factor, a TSH-treated group was always alternated with its saline-treated control group in the schedule of sacrifice.

After the intracardiac administration of 10 milliunits of TSH, the thyroidal concentration of water was unchanged for 10 minutes and then rose slightly in 20 minutes ($P < .05$) and more definitely in 30 minutes ($P < .001$). The maximal response was reached in 120 to 240 minutes and consisted of an increase of about 4 mg. of water per 100 mg. of thyroid weight, a rise of 5.5% (Fig. 1). Total wet weight of the thyroid tended to be higher after TSH than in the control groups, but not significantly so (Table 1). The increase in the concentration of water was the result both of an increase in water content and a decrease in the weight of solids per whole thyroid gland. The response of thyroidal water concentration of TSH

[2] The plasma water concentration observed in 52 chicks was 94.2 ± 0.12 mg. per 100 mg. Donnan factor was taken to be 0.95.

TABLE 1. THE EFFECT OF INTRACARDIAC TSH UPON THE WEIGHT, SOLIDS, AND WATER CONTENT OF THE CHICK THYROID

Experiment number	Substance injected	Time between TSH or saline and sacrifice (minutes)	Wet weight (mg./thyroid)	Dry weight (mg./thyroid)	Water (mg./thyroid)	Water concentration (mg./100 mg. thyroid)
13	Saline	30 and 240*	3.93 ± .089 (13)**	1.064 ± .022	2.87 ± .069	72.9 ± 0.20
	TSH***	30	4.07 ± .091 (9)	1.036 ± .020	3.04 ± .073	74.5 ± 0.22
	P-value, TSH versus saline		NS†	NS	NS	< .001
	TSH***	240	4.17 ± .061 (9)	0.999 ± .012	3.17 ± .053	76.0 ± 0.25
	P-value, TSH versus saline		NS	< .05	< .01	< .001
14	Saline	240	3.81 ± 0.11	1.130 ± .035	2.68 ± .076	70.3 ± 0.22
	TSH***	240	4.02 ± 0.12	1.036 ± .035	2.98 ± .089	74.2 ± 0.21
	P-value, TSH versus saline		NS	NS	< .05	< .001

* Six pools of animals received saline 30 minutes before sacrifice and seven were so treated 240 minutes before sacrifice. Since there were no differences between these two sub-groups, the pooled data are presented.
** Mean ± standard error of the mean (number of pools). Each pool consisted of the thyroid glands of 10 chicks in experiment 13 and 6 chicks in experiment 14.
*** TSH dose was 10 mU. in experiment 13, 100 mU. in experiment 14.
† NS—not significant (P > .05).

began to decline in 6 hours, was inconstant at 16 and 24 hours and had fully disappeared by 48 hours (Fig. 1).

Thyroidal Uptake of Radiosodium. Sodium-24 reached its maximum concentration in the thyroid gland very slowly in the absence of added TSH (Fig. 2 and Ref. 13, Table 1). The ultimate volume of distribution was suprisingly great; the 180-minute values defined volumes of distribution ranging from 46 to 51% of thyroidal weight and from 63 to 70% of the total thyroidal water. The phenomenon of delay in Na^{24} entry into the thyroid

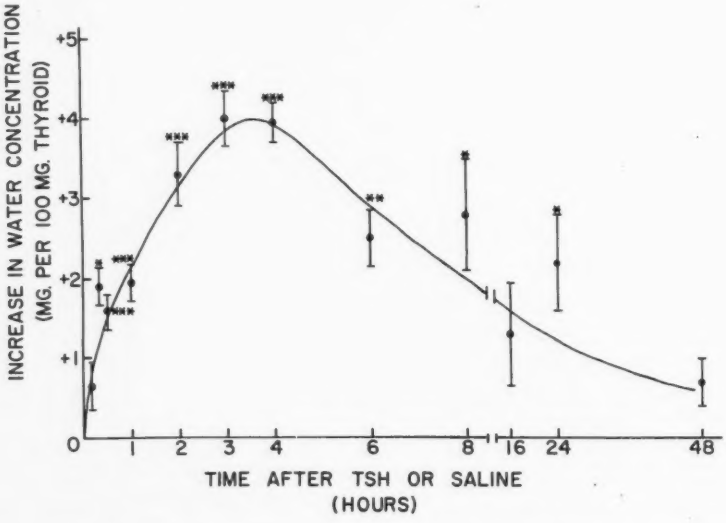

FIG. 1. The response of thyroidal concentration of water to TSH. Vertical lines in this and subsequent figures indicate one standard error of the mean. * = P < .05. ** = P < .01. *** = P < .001.

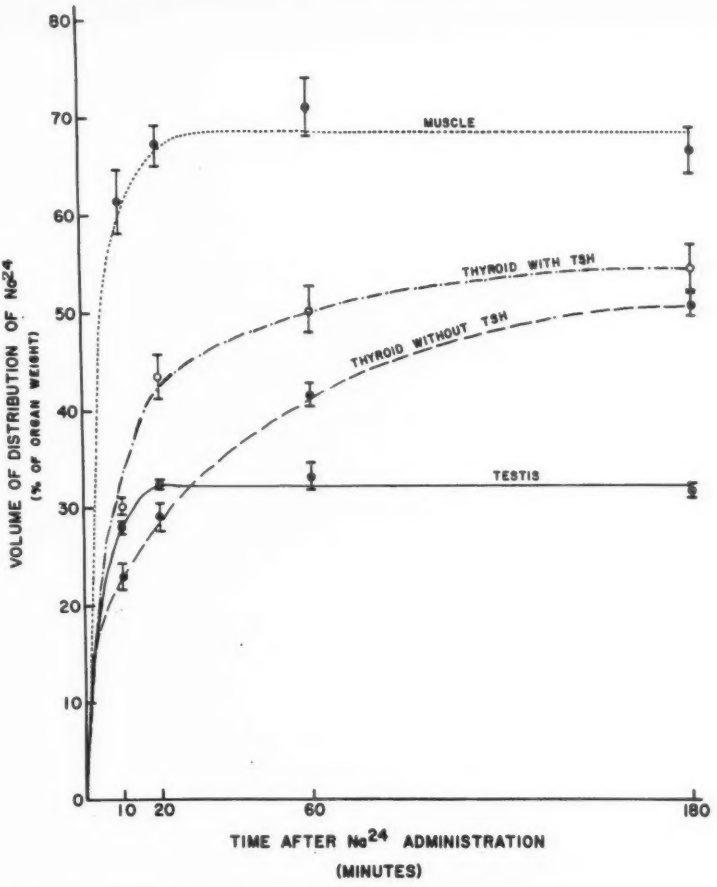

FIG. 2. The rapidity of movement of Na^{24} into various tissues of the chick. TSH was administered 20 minutes before Na^{24} in each group.

gland was confirmed by the fact that the volume of distribution 20 minutes after the injection of Na^{24} was significantly less than that at 180 minutes ($P < .001$) in each of three experiments. Even after 3 hours, the thyroidal concentration of radiosodium was not necessarily at its maximum, as shown in Experiment 12, Table 2, where the 5-hour and 24-hour volumes of distribution were significantly greater than that which existed at 3 hours. By contrast, Na^{24} reached its maximal distribution very rapidly in each of nine other tissues studied (Ref. 13, Table 1); the kinetics for all of these tissues are exemplified by those for muscle and testis shown in Figure 2. The 20-minute volume of distribution equalled that at 180 minutes in all instances except in the case of the duodenum, where a small but statistically significant increase was noted after 180 minutes.

TABLE 2. THE EFFECT OF TSH UPON THE VOLUME OF DISTRIBUTION OF Na^{22} IN THE CHICK THYROID, WHEN TSH IS ADMINISTERED AT LONG INTERVALS AFTER Na^{22}

Experiment number	Substance injected	Time between Na^{22} and TSH or saline injections (hours)	Time between TSH or saline injection and sacrifice (hours)	Volume of distribution of Na^{22}*	P-values: TSH versus saline	P-values: Saline versus previous saline
10	Saline	3	$\frac{1}{3}$	58.7 ± 1.73 (5)**		
	TSH	3	$\frac{1}{3}$	57.8 ± 1.99 (5)	NS†	
	Saline	3	1	59.9 ± 2.46 (6)		NS
	TSH	3	1	62.3 ± 1.69 (6)	NS	
	Saline	3	2	61.1 ± 0.75 (5)		NS
	TSH	3	2	62.1 ± 1.16 (5)	NS	
11	None	3	$\frac{1}{2}$	49.0 ± 0.67 (8)		
	Saline	3	$\frac{1}{2}$	49.4 ± 1.09 (8)		
	TSH	3	$\frac{1}{2}$	56.0 ± 1.79 (7)	< .01	
12	Saline	3	$\frac{1}{2}$	46.4 ± 1.57 (6)		
	TSH	3	$\frac{1}{2}$	53.1 ± 0.64 (6)	< .01	
	Saline	5	$\frac{1}{2}$	50.3 ± 0.92 (7)		< .05
	TSH	5	$\frac{1}{2}$	60.4 ± 1.42 (7)	< .001	
	Saline	24	$\frac{1}{2}$	56.2 ± 0.69 (7)		< .001
	TSH	24	$\frac{1}{2}$	59.6 ± 0.88 (6)	< .05	

* Volume of distribution in this and subsequent tables is expressed as a percentage of organ weight and is calculated as 100 times the Na^{22} per mg. of organ/Na^{22} per mg. of plasma.
** Mean ± standard error of the mean, with number of chicks in parentheses.
† See Table 1.

The administration of TSH accelerated the entry of Na^{24} into the thyroid gland (Fig. 2), while the ultimate volume of distribution was not significantly increased (difference between TSH-treated and saline-treated groups not significant at the 180-minute point in two experiments, barely significant at 0.05 level in a third). The enhancement by TSH of the 20-minute uptake of Na^{24} was significant in all three of these experiments and in several others. TSH produced no effect on the Na^{24} uptake of eight of the nine extrathyroidal tissues studied; in the case of the liver, there was an apparently significant reduction in the volume of distribution of Na^{24} in one experiment, but this was not confirmed in a second experiment.

The failure of TSH to enhance consistently the 180-minute thyroidal uptake of radiosodium might have been due to waning of the effectiveness of TSH during the longer time interval or to the fact that TSH acted only upon the rate of entry of radiosodium and not upon its ultimate volume of distribution. Accordingly, experiments were performed in which Na^{22} was administered many hours prior to the injection of saline or of TSH, and in which the animals were then sacrificed at short intervals thereafter, when TSH was known to be still operative (Table 2). In one instance (Experiment 10), the volume of distribution of radiosodium reached an apparent plateau in the saline-treated controls. In this situation, TSH caused no

further enlargement of the volume of distribution. When the volume of distribution of radiosodium had not reached a plateau before TSH was administered (Experiments 11 and 12), it was then significantly enhanced by TSH, as one would expect. In any case, when TSH was given *after* radiosodium, its apparent effect upon the thyroidal volume of distribution of the isotope was always very much smaller than when the hormone was given *before* the isotope (compare Fig. 2 with Table 2).

Since TSH altered principally the early rate of entry of Na^{24} into the thyroid gland, the 10-minute or 20-minute Na^{24} uptake was employed in all studies of the time-response and dose-response relationships. When TSH was administered only 1 minute before Na^{24}, no significant increase in 20-minute Na^{24} uptake was induced (Table 3), but a 5-minute interval resulted in a highly significant increase and 10-minute and 20-minute intervals gave maximal responses. By 60 and 100 minutes, the differences between the TSH-treated and saline-treated groups were barely significant, and at 220 minutes the effect was no longer significant.

When the interval between Na^{24} and sacrifice was shortened to 10 minutes, the latency of the effect of TSH proved to be even shorter than that suggested by the above data. An injection of TSH 5 minutes before the Na^{24}

TABLE 3. THE TIME-RESPONSE RELATIONSHIP FOR THE EFFECT OF TSH ON THE 20-MINUTE VOLUME OF DISTRIBUTION OF Na^{24} IN THE CHICK THYROID GLAND

Exper. No.	Substance administered	Time between TSH and Na^{24} injections (minutes)	Volume of distribution of Na^{24}*	P-value for difference between TSH and saline
6	Saline	1	25.9 ± 1.50 (6)**	
	TSH***	1	30.4 ± 3.69 (5)	NS†
9	Saline	5	27.3 ± 1.06 (6)	
	TSH	5	39.0 ± 2.71 (6)	< .01
7	Saline	10	27.9 ± 1.46 (6)	
	TSH	10	45.0 ± 1.49 (6)	< .001
7	Saline	20	28.2 ± 1.16 (6)	
	TSH	20	44.7 ± 1.78 (6)	< .001
6	TSH	20	46.6 ± 3.48 (6)	< .001††
6	Saline	60	30.1 ± 2.80 (5)	
	TSH	60	42.5 ± 4.43 (6)	< .05
6	TSH	100	32.3 ± 1.42 (6)	< .05††
6	Saline	220	24.8 ± 2.83 (4)	
	TSH	220	31.1 ± 1.34 (5)	NS†

* See Table 2.
** See Table 2.
*** TSH, 10 milliunits, or saline was administered by the intracardiac route at various times prior to the intravenous injection of Na^{24}. The interval between Na^{24} administration and sacrifice was in all cases 20 minutes.
† See Table 1.
†† t-test performed for this TSH-treated group against the pooled data of the three saline-treated groups of experiment 6. The pool had a mean of 27.0 ± 1.36 with an N of 15.

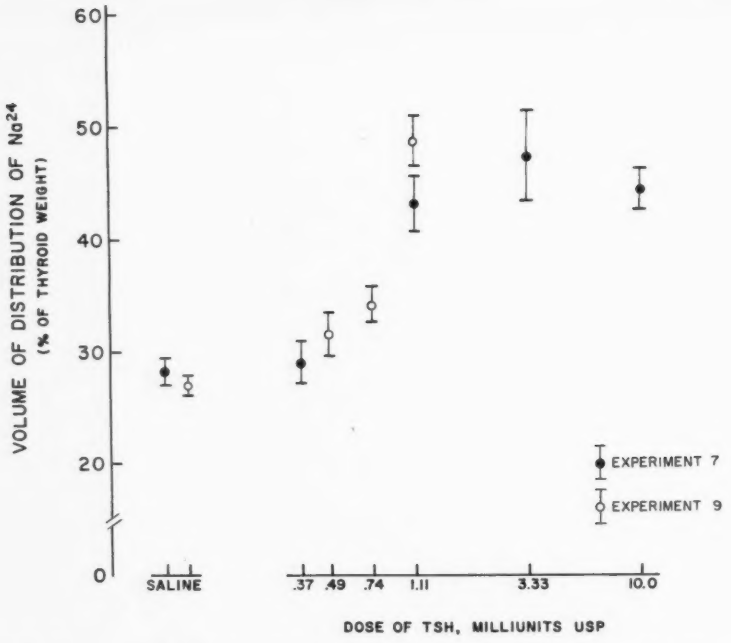

FIG. 3. Composite log dose-response curve for the effect of TSH on the 20-minute thyroidal volume of distribution of Na^{24}. TSH in each case was administered 20 minutes before Na^{24} and chicks were sacrificed 20 minutes after Na^{24}.

injection led to a small but significant increase in the volume of distribution of Na^{24}, and a 10-minute TSH-to-Na^{24} interval resulted in a highly significant increase.

The dose-response relationship is pictured in Figure 3. The threshold dose of TSH necessary for an effect on the 20-minute volume of distribution of Na^{24} was 0.49 U.S.P. milliunits; a dose of 0.37 milliunits was ineffective. TSH doses of 0.74 milliunits or higher induced responses which were significantly different from the saline group beyond the .001 level of probability. A maximal response was induced by 1.11 milliunits, defining a dose-response curve of steep slope, followed by a long plateau. Eighty micrograms of bovine albumin, an amount of protein approximately thrice that of the highest TSH dose, did not alter the volume of distribution of Na^{24}; nor did 500 U.S.P. milliunits of ACTH. Two additional preparations of TSH caused the same degree of response as did Thytropar® when administered in equal unitage. The first was U.S.P. Reference Standard; the second was a highly purified material prepared by Pierce *et al.* (14). The latter preparation contained approximately 15 U.S.P. units per mg., according to assay by chick P^{32} uptake (15) and human I^{132} uptake methods (16).

For comparison the dose-response characteristics of the acute effect of

TSH on the *early* phase of thyroidal P^{32} uptake was studied. When TSH was injected 20 minutes prior to P^{32}, the threshold dose for enhancement of the 20-minute uptake of P^{32} was greater than 1.25 milliunits, and a maximal effect was achieved with 5.0 milliunits. Hence, under identical temporal conditions, the radiosodium response appeared to be considerably more sensitive to TSH than was the response in P^{32} uptake.

With minor quantitative differences, all phenomena reported—the slow entry of radiosodium into the thyroid, the enchancement of the rate of entry by TSH, the time-response and dose-response relationships—were similarly clearcut when the radiosodium content was expressed in ways other than as volume of distribution; e.g., as total thyroidal radiosodium, radiosodium per gm. wet weight, radiosodium per gm. dry weight, radiosodium per gm. of thyroidal water, or volume of distribution of Na^{24} in thyroidal water.

Thyroidal Content of Stable Sodium and Potassium. The thyroid gland of untreated or saline-treated chicks contained 111 meq. of sodium and 60 meq. of potassium per kg. of fresh tissue. Together, these data dictated a cation concentration of greater than 236 meq. per kg. of thyroidal water, (Table 4). The concentration of both cations in muscle was lower than that in thyroid, whether expressed per kg. of fresh tissue or per kg. of tissue water. Plasma contained 148 meq. per L. of sodium and 5.2 meq. per L. of potassium.

The administration of TSH did not increase the content or concentration of stable sodium, when studied either 30 minutes after TSH injection, a time when the increase in thyroidal radiosodium uptake was maximal, or 240 minutes after TSH injection, a time when the increase in thyroidal water concentration was maximal (Table 4). Instead, an actual *decrease* occurred, which was significant in the 240-minute group. The concentration of potassium, on the other hand, did not change significantly after 30 minutes, but *rose* significantly after 240 minutes. The latter increase was partly attributable to the increase in water content manifest at that time; however, potassium concentration per kg. of tissue water was also significantly elevated. The total concentration of both cations in thyroidal water fell significantly 30 minutes after TSH administration; after 240 minutes, the fall in sodium was balanced by the rise in potassium. TSH caused no change in cation concentrations in muscle or in plasma.

Technical problems made it difficult to determine radiosodium and stable sodium values on the same thyroid glands; hence no figures for true specific activity are available. However, the findings regarding radiosodium, sodium, and water concentrations were so consistent among many different experiments that it seemed reasonable to calculate specific activities and sodium spaces from the mean values of different experiments. Such derived data are presented in Table 5. It should be noted that the specific activity of thyroidal sodium is considerably less than that of plasma sodium, even

TABLE 4. THE EFFECT OF TSH ON THE CONCENTRATION OF STABLE SODIUM AND POTASSIUM OF THYROID AND MUSCLE TISSUE IN THE CHICK

Substances administered	Number of pools*	Water, gm. per kg. fresh tissue	Sodium		Potassium	
			meq. per kg. fresh tissue	meq. per kg. tissue water	meq. per kg. fresh tissue	meq. per kg. tissue water
Thyroid						
Saline**	10	728 ± 2.0	111 ± 5.0***	153 ± 7.1	60 ± 3.1	83 ± 4.2
TSH, 30 min.	8	744 ± 2.1	96 ± 6.6	129 ± 9.2	54 ± 2.5	73 ± 3.3
p-value, TSH versus saline		< .001	NS†	NS	NS	NS
TSH, 240 min.	8	760 ± 2.7	94 ± 5.6	124 ± 6.4	73 ± 1.5	96 ± 2.0
p-value, TSH versus saline		< .001	< .05	< .02	< .01	< .05
Muscle						
Saline	8	844 ± 3.6	96 ± 3.2	113 ± 3.8	41 ± 1.5	49 ± 2.1
TSH, 30 or 240 min.	7	843 ± 2.5	93 ± 1.2	110 ± 1.6	41 ± 1.9	49 ± 2.4
p-value, TSH versus saline		NS	NS	NS	NS	NS

* Each pool consisted of the thyroid glands of ten chicks or the pectoral muscle of two of the same chicks.

** Saline was administered either 30 minutes or 240 minutes prior to sacrifice. Since there was no difference between the two sub-groups, the pooled data are presented.

*** See Table 2.

† See Table 1.

when 24 hours have been allowed for exchange to occur. By contrast, the specific activity of muscle sodium attains equality with that of plasma in only 20 minutes.

Thyroidal Uptake of Radio-iodinated Human Serum Albumin: In order to

TABLE 5. COMPARISON OF SPACES CALCULATED FROM ISOTOPIC AND STABLE-ELEMENT MEASUREMENTS OF THYROID AND MUSCLE†

	Per kg. fresh tissue			
	Thyroid, control	Thyroid, 30 minutes after TSH**	Muscle	Plasma
Water (gm.)	723	744	843	942
Na (meq.)	111	96	96	144
K (meq.)	60	54	41	5.0
Na and K (meq.)	171	150	137	149
20-minute Na* (% admin. dose)	1638	2634	4099	6060
20-minute S.A. (% admin. dose/meq.)	14.8	27.4	42.7	42.1
24-hour Na* (% admin. dose)	2772	—	—	4890
24-hour S.A. (% admin. dose/meq.)	25.0	—	—	34.0
20-minute Na* space (gm.)	268	432	666	—
24-hour Na* space (gm.)	558	—	—	—
Na space (gm.)	765	662	662	—

† Original data are derived from the experiments described in Tables 1, 2, and 4, this paper, and in Table 1, Ref. 13.

** In isotopic experiments, TSH was administered 10 minutes before Na* and 30 minutes before sacrifice.

Na* = radioactive Na.

S.A. = specific activity.

TABLE 6. THE EFFECT OF TSH UPON THE 10-MINUTE THYROIDAL VOLUME OF DISTRIBUTION OF RADIO-IODINATED SERUM ALBUMIN AND UPON THE 10-MINUTE THYROIDAL UPTAKE OF RADIO-IODIDE

Radio-iodinated human serum albumin			
Substance administered	Time between TSH and RISA injections (minutes)	Volume of distribution of RISA*	P-value for difference between TSH and saline***
Saline	5	5.60 ± 0.16 (6)**	—
TSH‡	5	4.76 ± 0.30 (6)	< .01
TSH	10	5.56 ± 0.19 (6)	NS†
TSH	20	6.18 ± 0.39 (6)	NS
TSH	60	6.12 ± 0.23 (6)	NS
TSH	180	6.73 ± 0.35 (6)	< .01
Saline	180	5.88 ± 0.18 (6)	—
Radio-iodide			
Substance administered	Time between TSH and I^{131} injections (minutes)	Thyroidal uptake of I^{131} (per cent of administered dose)	P-value for difference between TSH and saline**
Saline	1	.202 ± .0205 (6)	—
TSH‡	1	.183 ± .0213 (5)	NS
TSH	10	.223 ± .0291 (6)	NS
TSH	20	.233 ± .0289 (6)	NS
TSH	40	.302 ± .0716 (6)	NS
Saline	60	.237 ± .0201 (5)	—
TSH	60	.197 ± .0117 (5)	NS
TSH	120	.246 ± .0270 (5)	NS
Saline	180	.220 ± .0223 (6)	—
TSH	180	.391 ± .0553 (6)	< .01

* See Table 2.
** See Table 2.
*** In both experiments the saline groups were not significantly different from each other (by analysis of variance) and were therefore pooled for comparison with TSH groups.
† See Table 1.
‡ TSH dose in RISA experiment was 10 milliunits; in I^{131} experiment it was 25 milliunits.

study intrathyroidal plasma volume, chicks were sacrificed ten minutes after the administration of RISA. In untreated chicks the intrathyroidal albumin space at that interval was 5.74% of organ weight. TSH administered 5 minutes earlier apparently reduced the volume of distribution of RISA (Table 6). TSH administered 10, 20 or 60 minutes before RISA had no effect on the volume of distribution; only when TSH was given 180 minutes before RISA was there an increase in intrathyroidal radioactivity. However, by this time, TSH had begun to enhance thyroidal uptake of radioiodide (Table 6) and hence these later results are no longer necessarily indicative of an effect on thyroidal albumin space. It is to be emphasized that TSH did not increase 10-minute radioiodide uptake until after a latency of greater than 120 minutes. Thus, even if significant amounts of free iodide were being released from RISA, constancy of thyroidal I^{131} with and without TSH must be interpreted as indicating no enhancing effect of TSH on the thyroidal volume of distribution of albumin.

After administration of TSH, there was no visible reddening or alteration in color of the thyroid gland at any of the time intervals studied.

DISCUSSION

The thyroid gland of the untreated newly-hatched chick exhibits certain unusual chemical characteristics. The first is a high ratio of sodium to potassium; this is characteristic of immature tissues in general and has been interpreted as indicating inefficiency of sodium extrusion mechanisms (17, 18 and Table 4). In several species a rapid decline in the sodium and chloride of muscle occurs soon after birth, while the potassium content is climbing to adult levels (18, 19). However, the high sodium/potassium ratio probably persists in thyroid gland despite maturation, since Keller found a ratio of 1.6/1.0 in a few analyses of adult sheep thyroids (20). Further studies on this matter are in progress in our laboratory. Secondly, the total thyroidal concentration of the two cations is extraordinarily high, amounting to 236 meq. per kg. of tissue water. The observed sodium cannot all be assigned to the interstitial spaces, since this compartment would thereby have to amount to 76 % of organ weight or over 100 % of thyroidal water. Nor can any reasonable estimate for intracellular cation concentration account either for the excess sodium or for all of the thyroidal potassium (see Appendix). A dissection of thyroidal cation distribution, based on more reasonable assumptions, indeed reveals that approximately 250 meq. of sodium and 80 meq. of potassium exist in the remaining compartment, the follicular lumen, per liter of colloid water (see Appendix). This defines an "extra" extracellular space, the follicular lumen, rich in protein and cations and poor in water. From data reported in summary only by Hastings, it is clear that considerable chloride is also present in the colloid (21). Thus, we can infer at least some degree of permeability of the apical cell membrane to sodium, potassium and chloride. On the other hand, it is presumably impermeable to thyroglobulin, which has an isoelectric point of 4.6 (22) and is dissociated as an anion at the pH of 6.6 observed for colloid (23). With a large content of an impermeable anion, one might have a high concentration of cations as a result of the Gibbs-Donnan effect and still maintain osmotic stability (24). However, the sum of the estimated intraluminal concentrations of sodium and potassium alone exceeds the total osmolar concentration usually present in intracellular and extracellular fluids (Appendix). It thus seems probable that a considerable portion of the intraluminal cation exists in salt-linkage with protein rather than as osmotically active ions.

The temporal course of the entry of radiosodium into the thyroid gland of the untreated newly-hatched chick lends firm support to the concept that sodium occupies a space larger than that characterized as interstitial. Among the ten organs studied, only the thyroid showed a concentration of radioisotope which rose progressively for many hours. In all others, a steady

state was achieved (assuming no alteration in stable sodium content) within 20 minutes. This was true even in muscle, where the extracellular (or, more accurately, the rapidly-equilibrating) space was very large, 66% of organ weight, and where the radiosodium and sodium spaces were identical (Table 5). Since there is no reason to postulate the existence of a barrier to sodium transfer across the thyroidal capillary membrane, the slow rate of entry of the isotope appears to indicate a delay in sodium transfer across one or more structures of cellular origin. In view of the presence of sodium in the intraluminal colloid, the barriers presumably include both the basal and the apical cell membranes.

An alternative possibility is that sodium enters colloid through intercellular channels, but that equilibration with the highly viscous follicular contents is by slow diffusion. Intercellular channels were postulated many years ago as a route for egress of thyroidal secretion (25); however later studies by light and electron microscopy have essentially excluded their presence as anatomical entities (26). It thus seems far more likely that sodium must traverse cellular membranes in order to reach the follicular lumen. However, once there, the factor of slow diffusion toward the center of the gelatinous colloid may well be involved; it would appear to be the only reasonable explanation of the fact that thyroidal radiosodium space is still expanding between 5 and 24 hours after isotope administration in one experiment and especially of the fact that this space even after 24 hours is considerably smaller than the stable sodium space (55–60% of organ weight as compared to about 75%; see Table 5).

The response to the administration of TSH is divisible into early effects (30 minutes) and later effects (240 minutes). The early action is highlighted by a sudden expansion of the 10-minute volume of distribution of radiosodium. This phenomenon might theoretically be due to (1) an expansion of the thyroidal vascular sodium pool, (2) an expansion of the thyroidal interstitial sodium pool, or (3) a heightened rate of penetration of radiosodium into more slowly-equilibrating compartments, presumably the intracellular and intraluminal contents. The first possibility is made unlikely by the fact that TSH, under identical temporal conditions, does not increase the volume of distribution of RISA; this finding also tends to minimize the likelihood that any sizeable change is initiated in local capillary permeability. Regarding the second possibility, a significant expansion of the interstitial sodium pool *with plasma sodium as its source* could not reasonably have occurred in view of the available data. Such an event would have to be accompanied by an increase in the total thyroidal content of stable sodium and by a sizeable increase in the radiosodium space when radiosodium had nearly equilibrated before TSH was administered. Assuming free transfer of water across the capillary, an increase in thyroidal water concentration from 72.3% to 76.5% would also be predicted. At short time intervals after TSH administration, when the increase in radiosodium uptake is maximal,

none of these occurrences is observed. Expansion of the interstitial sodium pool as a result of a net transfer of sodium from the intraluminal and intracellular compartments is, on the other hand, a plausible explanation of the observed effects of TSH. This rearrangement would require no increase in total thyroidal content of stable sodium, of pre-equilibrated radiosodium or of water. Plasma radiosodium of high specific activity would rapidly exchange with the enlarged pool of interstitial sodium, thereby accounting for the enhancement of the early phase of thyroidal radiosodium uptake induced by TSH.

Equally plausible is the third proposed action of TSH, i.e., induction of a primary increase in the permeability of those cellular structures which presumably constitute a barrier to sodium transfer. If this is the case, it should be emphasized that no *net* inward transfer occurs, since free access to plasma sodium would then lead to an increase in stable sodium content of the organ as a whole, rather than the observed decrease. If anything, stimulation of efflux somewhat in excess of influx must be postulated. Direct measurement of fluxes, with and without TSH, must await *in vitro* studies in which sampling at frequent time intervals is possible.

Both of the hypotheses which acceptably explain the observed data involve the assumption of enhanced permeability of cell membranes to sodium. The mechanism for this enhancement is entirely unknown. Possibilities include activation of the cellular sodium-extrusion pump (presumably in the centrifugal direction), acceleration of passive bidirectional ion-transfer mechanisms, or enhancement of the transfer of sodium in the form of salts or amino acids derived from thyroglobulin.

The rapidity of onset of the observed responses to TSH administration is of some importance; the effect on water content begins in 20 minutes and that on radiosodium uptake in 15 minutes or less. There are no data in the literature on the latency of various thyroidal responses to intravenously administered TSH in the chick. TSH administered by other routes does not induce changes in various criteria of thyroidal response until latencies of one hour or more (27, 28). In our own studies, neither P^{32} uptake, nor I^{131} uptake, nor the release of thyroidal radioiodine into the plasma is initiated as rapidly as is the increase in radiosodium uptake (unpublished observations). From this fact and from the very low threshold dose of TSH required to elicit the radiosodium response, one may suggest that the effect of TSH on thyroidal radiosodium uptake is in some way an indicator of the primary action of the hormone on its target gland.

If the primary action of TSH were an activation of the rate of proteolysis of thyroglobulin (23, 29), the accompanying osmotic and electrochemical changes might well initiate secondary changes in radiosodium distribution, as suggested by Bakke *et al.* to explain water shifts (11). It is curious, however, that the effect on radiosodium uptake is easily demonstrable approximately 25 minutes before an efflux of iodinated amino acids is detectable in

the blood (unpublished observation). This lack of simultaneity, if not simply due to technical factors, could be explained by assuming that proteolysis must proceed through several stages before amino acids actually appear in the blood, a concept for which there is considerable suggestive evidence from enzymatic studies (30). The accumulation of water noted by Bakke *et al.* to occur in beef thyroid slices in response to very small amounts of TSH added *in vitro* appeared to be located in the follicular lumina, as would be expected if it were secondary to proteolysis. However, it is difficult to compare the *in vitro* preparation with that described in the present study, since the former exhibited water-imbibition only after a prolonged latent period.

On the other hand, it is equally possible that the primary action of TSH is directed toward aspects of thyroidal metabolism quite distinct from hormonogenesis or thyroglobulin-lysis. Freinkel presented evidence that TSH stimulates oxygen consumption and the incorporation of P^{32} into phospholipid in thyroid slices *in vitro*, with temporal characteristics suggesting almost instantaneous onset of the effects (31). He suggested that TSH might primarily stimulate intermediary metabolism of thyroid tissue, with both growth and enhancement of hormonogenesis following secondarily. Additional evidence for this concept has been provided by the demonstration of stimulation of pathways of glucose metabolism *in vitro* by TSH (32, 33). In the light of this hypothesis, the shifts in water and sodium demonstrated in the present study may result from alterations in membrane permeability which in turn depend on an enhancement in intracellular energy production. Such a sequence would be expected if the sodium-pump were activated, since the integrity of this mechanism is clearly dependent on cellular energy metabolism (34). As mentioned previously, enhancement by TSH of the extrusion of sodium from the "inner" compartments of the thyroid follicle to the interstitium is a process quite compatible with our observed data.

Finally, TSH might primarily alter the permeability of the cell membrane and perhaps of intracellular structures as well, an action in line with that proposed for hormones in general by Hechter (1). The resulting rearrangement in the local concentrations of substrates, of substrate-carriers and of enzymes might well lead secondarily to the stimulation of organic metabolism noted by Freinkel, Field and others. According to this concept, the accelerated flux of sodium and the accumulation of water observed in our studies would simply be indicators of a much more general alteration in permeability. Under current investigation is the possibility of alterations in the intrathyroidal distribution of other inorganic and organic substances under the influence of TSH.

The effects of TSH observed after four hours have elapsed are quite different from the earlier results of hormonal administration. There is no longer any acceleration of the rate of entry of radiosodium into the thyroid.

An actual fall in the concentration of stable sodium has occurred, balanced by an increase in potassium, while the water concentration has reached its maximal increase. By four hours after TSH administration, an increase in cellular volume and a discharge of colloid are well established phenomena in the chick (27). The observations described herein are quite compatible with an enlargement of the potassium-rich intracellular space and a decrease in the volume of the sodium-rich and water-poor intraluminal space. An exchange of potassium for sodium is implied, but further studies will be needed to analyze this point.

Addendum

After this paper was submitted, we became aware of the publication by Gedda, P.O., Acta endocrinologica, Suppl. No. 56, Jan. 1961. He reports that, in the guinea pig, TSH increases the wet weight and potassium concentration of the thyroid in 2 hours. Increase in sodium concentration and iron content in 2 hours were also reported, but the observed changes were not significant by t-test. No isotopic studies were reported.

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APPENDIX

Estimation of Intraluminal Concentration of Electrolytes

Symbols:

( ) = concentration per unit volume (meq. or gm. per L.)
[ ] = concentration per unit weight (meq. or gm. per Kg.)
{ } = concentration per unit weight of water (meq. or gm. per Kg.)
W = weight of a compartment (Kg. per Kg. of organ)
Na, K, H_2O without brackets = quantity (meq. or gm.)
Subscripts: I = interstitial fluid
C = cells
L = luminal contents
P = plasma
T = thyroid

Observations:

$[Na]_T$ = 111 meq./Kg. thyroid
$[K]_T$ = 60 meq./Kg. thyroid
$[H_2O]_T$ = 723 gm./Kg. thyroid
$(Na)_P$ = 148 meq./L. plasma
$(K)_P$ = 5.2 meq./L. plasma
$[H_2O]_P$ = 942 gm./Kg. plasma

Assumptions:

Gibbs-Donnan factor, plasma-to-interstitial fluid = 0.95
Density of plasma = 1030 gm./L.

W_I = 0.15
W_C = 0.43 (Ref. 23)

$W_L = 0.42$ (Ref. 23)
$\{Na\}_C =$ 69 meq./Kg. cell water (calculated from data on muscle from Table 7 and Reference 17)
$\{K\}_C = 125$ meq./Kg. cell water (calculated from data on muscle from Table 7 and Reference 17)
$[H_2O]_C = 700$ gm./Kg. thyroid cells

Calculations:

A. *Intraluminal sodium content:*

1) $[Na]_P = \frac{(Na)_P}{1.030} = 144$ meq./Kg.

2) $[Na]_I = \frac{[Na]_P(0.95)}{(0.942)} = 145$ meq./Kg.

3) $Na_I = [Na]_I(W_I)$
$= (145)\ (0.15)$
$= 21.8$ meq.

4) $Na_C = \frac{Na_C[H_2O]_C W_C}{1000}$
$= \frac{(69)\ (700)\ (0.43)}{1000}$
$= 29.8$ meq.

5) $Na_L = Na_T - Na_I - Na_C$
$= 111 - 21.8 - 20.8$
$= 68.4$ meq.

B. *Intraluminal potassium content:*

1) $[K]_P = \frac{(K)_P}{1.030} = 5.0$ meq./Kg.

2) $[K]_I = \frac{(5.0)(0.95)}{0.942} = 5.1$ meq./Kg.

3) $K_I = (5.1)(0.15) = 0.8$ meq.
4) $K_C = (125)(0.301) = 37.6$ meq.
5) $K_L = K_T - K_I - K_C$
$= 60 - 0.8 - 37.6$
$= 21.6$ meq.

C. *Intraluminal water content:*

1) $H_2O_I = 150$ gm.
2) $H_2O_C = [H_2O]_C(W_C)$
$= (700)(0.43)$
$= 301$ gm.
3) $H_2O_L = H_2O_T - H_2O_I - H_2O_C$
$= 723 - 150 - 301$
$= 272$ gm.

D. *Intraluminal sodium and potassium concentrations:*

1) $[Na]_L = \frac{Na_L}{W_L} = \frac{68.4}{0.42} = 163$ meq./Kg. luminal contents.

2) $\{Na\}_L = \frac{1000\ Na_L}{H_2O_L} = \frac{68{,}400}{272} = 251$ meq./Kg. luminal water.

3) $[K]_L = \frac{K_L}{W_L} = \frac{21.6}{0.42} = 51.5$ meq./Kg. luminal contents.

4) $\{K\}_L = \frac{1000\ K_L}{H_2O_L} = \frac{21{,}600}{272} = 79.4$ meq./Kg. luminal contents.

5) $\{Na+K\}_L = 251+79 = 330$ meq./Kg. luminal water.

6) $[H_2O]_L = \frac{272}{420} \times 1000 = 648$ gm./Kg. luminal contents.

Notes: Of the many assumptions involved in the above estimates, the choice of the values for W_I, W_C and W_L is the most important in terms of materially changing the final concentrations. From the histologic observation that "stroma" in the one-day-old chick thyroid is almost negligible (27), one might guess that W_I may be even smaller than 0.15. On the other hand a space equal to 20–25% of organ weight exchanges rapidly with radiosodium. Furthermore, intrathyroidal plasma volume is 5.7% of organ weight, and interstitial volume is often about three times an organ's plasma volume. Hence 0.15 seems to be a reasonable compromise. If a lower figure is taken for W_I, and the ratio of W_C to W_L is held constant, one would derive a lower value for $\{Na_L+K_L\}$. However, even at $W_I = 0$, $\{Na_L+K_L\}$ would still be 278 meq. per Kg. of luminal water. Values of W_I greater than 0.15 would lead to values for $\{Na_L+K_L\}$ even greater than the estimated one of 330 meq. per Kg. of water.

Lamberg and Olin-Lamberg (28) found the epithelial volume of the young chick thyroid to be higher than did Wahlberg, whose figures were used above as a basis for choosing W_C and W_L. If we hold W_I constant at 0.15 and set W_C equal to 0.68 (any value higher than this would give a negative figure for K_L), then $\{Na+K\}_L$ would rise to 580 meq. per Kg. of intraluminal water.

The values of $\{Na\}_C$, $\{K\}_C$ and $[H_2O]_C$ appear reasonably accurate for muscle cells, since they are based both on our own figures and on those of Barlow and Manery (17). There was excellent agreement between the two sets of results. To apply these estimates to thyroid cells is obviously hazardous, but the water concentration, Na/K ratio and total osmolar concentration all appear to be reasonable values for tissue cells of immature animals. To refine these estimates further will require direct analysis of the contents of thyroid follicles.

THE EFFECT OF TOTAL AND SUBTOTAL ADRENALECTOMY ON THE RENAL AND EXTRA-RENAL RESPONSE OF THE DOMESTIC DUCK (*ANAS PLATYRHYNCHUS*) TO SALINE LOADING[1]

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ABSTRACT

Total adrenalectomy of the saline loaded domestic duck resulted in complete obliteration of the extra-renal excretory response. Subtotal adrenalectomy resulted in a diminished response to the saline load compared to similarly treated intact control birds. The maintenance of adrenalectomized ducks on cortisol restored the extra-renal excretory pattern to near normal levels.

The significance of this, and previous investigations of the extra-renal process in marine birds, is discussed.

THE extra-renal excretion by marine birds of orally administered sodium chloride has been shown to be associated with the paired supra-orbital glands which discharge a highly concentrated solution of electrolytes into the anterior nasal cavity (1, 2, 3, 4, 5).

Recent work (5) involving the simultaneous administration of certain adrenocortical steroids along with the saline load indicated an enhancement of the rate and quantity of nasal discharge from domestic ducks. Moreover, the nasal secretion appeared to be independent of the osmotic stimulus in the presence of these exogenous hormones.

In the present work an attempt was made to correlate more closely the dependence of the extra-renal excretory mechanism on an intact and functioning adrenal cortex. To this end the effect of total and subtotal adrenalectomy on the nasal discharge were studied under exactly the same experimental conditions as those employed during our previous investigations (5).

MATERIALS AND METHODS

All birds used throughout these experiments were 9- to 12-week-old male domestic ducks of the Pekin white variety. They were fed daily with commercial "duck grower

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food" and given an *ad libitum* supply of fresh water. The ten control birds and each experimental group of 5 animals were starved for 24 hours before use. A wing vein was cannulated and the bird was heparinized 1½ hours prior to the commencement of the experiment. A sheet of polyethylene was placed under the cloacal region and the bird was fastened in a ventral position to a standard "dog board" by means of rubber loops over the wings and legs. The sheet was pulled down to form a funnel in the centre of the "dog board" and a hole was cut in the bottom of this funnel for urine collection.

Saline loading was by means of a flexible plastic tube connected to a syringe and 20 ml. of 20% NaCl (68.97 m.eq. of Na^+) was introduced directly into the glandular stomach (proventriculus). The time of saline loading was taken to be zero time for all experiments.

A control blood sample was taken from the wing vein prior to the saline loading and, subsequent to loading, samples were taken at ½ hourly intervals for the first 3 hours and at hourly intervals for the remaining experimental period. The blood was immediately centrifuged at 2° C and 5000 rpm for 5 minutes. Urine samples were collected and measured at 20 minute intervals after zero time. The time of onset of nasal secretion was recorded and collections were made over 45 minute periods thereafter.

Adrenalectomy was performed via a dorso-lateral incision between the second and third ribs. Bilateral adrenalectomy was done in two stages on successive days and the birds were used experimentally 6 to 12 hours after removal of the second gland. Unilateral adrenalectomy was performed approximately 12 hours before saline loading. After this experimental period the unilaterally adrenalectomized birds were intramuscularly injected with 10 mg. of cortisol in stabilized suspension, and the second adrenal gland was removed. These totally adrenalectomized ducks were then maintained for 4 days with daily intramuscular injections of 5 mg. of cortisol. The saline loading was then repeated with these birds but serial blood samples were not taken during this experimental period.

At autopsy the adrenalectomized birds were examined for adrenal tissue.

A Zeiss PF 5 flame photometer was used for all sodium and potassium estimations of plasma, urine and nasal samples. Electrolyte values were expressed in milliequivalents/litre.

RESULTS

As previously reported (5), the outstanding feature of the response by the intact duck to a saline load was an initial diuresis during the first hour followed by an extra-renal phase of secretion. This extra-renal phase commenced almost simultaneously with the cessation of diuresis.

Renal Phase: Table 1

The mean total diuresis by the control birds during the three successive 20 min. collection periods was 51.9 ± 3.2 cc. The individual volume declined with each successive sample from 23.3 ± 4.8 cc. in the first to 11.5 ± 4.2 cc. in the third. There was also a decline in the sodium ouput in these samples from 14.8 ± 6.2 m.eq. to 2.4 ± 1.6 m.eq. This decline was disproportionate to the reduction in volume as indicated by the mean sodium concentrations of these samples (633 ± 70.4 vs 248 ± 115.6 m.eq./l.) which were significantly different ($P < .01$). Conversely the potassium concentrations appeared to rise from 13.6 ± 2.8 m.eq./l. in the first sample to 24.7 ± 12.2 m.eq./l. in the third, but this change was not statistically significant.

Unilateral adrenalectomy 12 hours prior to saline loading did not have

TABLE 1. THE ANALYSIS OF SUCCESSIVE 20-MINUTE URINE SAMPLES FROM TOTALLY AND SUBTOTALLY ADRENALECTOMIZED DOMESTIC DUCKS SUBSEQUENT TO THE ADMINISTRATION OF A SALINE LOAD

	Urine sample	Total renal output in m.eq.				Urine concentration in m.eq./liter	
		Volume in cc.	Na^+	K^+	$Na^+:K^+$	Na^+	K^+
Intact Control	1	23.3 ±4.8	14.8 ±6.2	0.27 ±0.02	49.2 ±6.3	633.0 ±70.4	13.6 ±2.8
	2	17.1 ±4.3	7.7 ±1.8	0.17 ±0.06	52.1 ±9.2	455.8 ±34.8	11.0 ±7.7
	3	11.5 ±4.2	2.4 ±1.6	0.27 ±0.23	46.3 ±16.5	248.0 ±115.6	24.7 ±12.2
	Total	51.9 ±3.2	24.9 ±2.9	0.71 ±0.23	47.4 ±8.8	482.2 ±24.1	12.7 ±3.5
Unilateral Adrenalectomy	1	19.5 ±11.1	11.8 ±3.9	0.84 ±0.53	16.42 ±5.6	683.3 ±67.4	38.9 ±5.4
	2	23.0 ±5.8	8.3 ±2.8	0.28 ±0.07	36.40 ±9.2	339.6 ±48.1	11.76 ±2.5
	3	1.2*	0.1	0.20	1.54	91.7	59.5
	Total	43.7 ±12.1	20.2 ±5.9	1.32 ±0.52	20.19 ±5.3	398.5 ±69.2	29.8 ±7.8
Total Adrenalectomy	1	22.9 ±8.5	14.7 ±6.5	0.44 ±0.19	32.27 ±8.2	595.9 ±79.5	32.3 ±14.4
	2	25.8 ±6.8	13.6 ±4.7	0.33 ±0.12	50.92 ±9.8	505.6 ±52.1	12.3 ±3.5
	3	10.5 ±4.5	3.6 ±1.5	0.13 ±0.06	37.63 ±16.4	398.3 ±84.4	8.7 ±1.2
	4+	23.1 ±9.6	2.5 ±2.0	0.18 ±0.08	13.29 ±2.3	65.7 ±15.8	7.5 ±0.5
	Total	82.3 ±4.8	34.4 ±3.5	1.08 ±0.07	32.82 ±4.5	478.3 ±85.7	16.7 ±4.2
Total Adrenalectomy +Cortisol	1	37.1 ±12.9	16.9 ±5.5	0.43 ±0.15	45.73 ±11.6	564.0 ±184.7	12.4 ±2.0
	2	8.1 ±4.4	1.6 ±0.5	0.15 ±0.10	27.57 ±15.2	221.4 ±83.1	14.7 ±3.8
	3	—	—	—	—	—	—
	4+	1.8*	0.2	0.04	4.32	95.6	21.0
	Total	47.0 ±9.4	18.7 ±5.1	0.62 ±0.02	34.72 ±12.2	437.8 ±153.0	14.8 ±2.6

* Only one urine sample from one animal.

any significant effect on either the total volume of urine or the total sodium and potassium excreted. The phase of diuresis, however, was shorter in 4 of the birds; there being a third urine sample in only one duck.

Total adrenalectomy considerably extended the period of diuresis subsequent to saline loading. The degree of extension varied from 1 to 15 twenty-minute urine samples with a mean number of 6.6 samples for the five birds in the group. The results of the analysis of urine samples from the fourth collection period and later have been pooled for each bird and are indicated as mean values of sample "4+" in Table 1. The total volume of urine excreted by the adrenalectomized ducks was 82.3 ± 4.8 cc. which was very significantly higher than the control value of 51.9 ± 3.2 cc. ($P < .001$). The total sodium excreted was 34.4 ± 3.5 m.eq. which was higher than the control output at somewhat less than the 10% level of significance ($P < .1, > .05$). Potassium excretion appeared to be unchanged when compared to the controls.

Maintenance of the adrenalectomized birds with exogenous cortisol significantly reduced the volume of urine excreted to 47.0 ± 9.4 cc. This value was significantly lower than the high rate of diuresis recorded for the adrenalectomized birds ($P < .02, > .01$) but did not differ significantly from the controls ($P > .5$). Similarly there was a significant renal retention of sodium since the total output was 18.7 ± 5.1 m.eq. compared to 34.4 ± 3.5 m.eq. after total adrenalectomy ($P < .05, > .025$). This total sodium output, however, did not vary significantly from the control output ($P > .2$). No significant effect of cortisol could be demonstrated with respect to potassium excretion.

Extra-renal Phase: Table 2, Figure 1

The volume of nasal secretion collected from intact control birds during the first 45 minutes after the onset was 1.58 ± 0.42 cc. and rose to a maximum value of 4.95 ± 0.56 cc. during the third collection period (90–135). The subsequent decline in output resulted in a complete cessation in most animals during the final period (450–495 min.). The sodium and potassium concentrations of successive nasal samples did not vary significantly and the total volume secreted (28.9 ± 3.5 cc.) contained 17.1 ± 1.6 m.eq. of sodium and 0.44 ± 0.06 m.eq. of potassium. These mean outputs indicated concentrations of sodium and potassium in the nasal discharge of 602.6 ± 13.9 and 17.3 ± 0.5 m.eq./l. respectively.

During the first 45-minute collection period the unilaterally adrenalectomized ducks showed a nasal output of 4.35 ± 1.01 cc. This initial output was the highest for any collection period and significantly higher than the corresponding control value ($P < .05, > .02$) but there was a decline in output after the first 45 minutes resulting in complete cessation in all birds 315 min. after loading. The mean total output of 15.5 ± 3.9 cc. contained 8.6 ± 2.2 m.eq. of sodium and 0.21 ± 0.05 m.eq. of potassium. All these

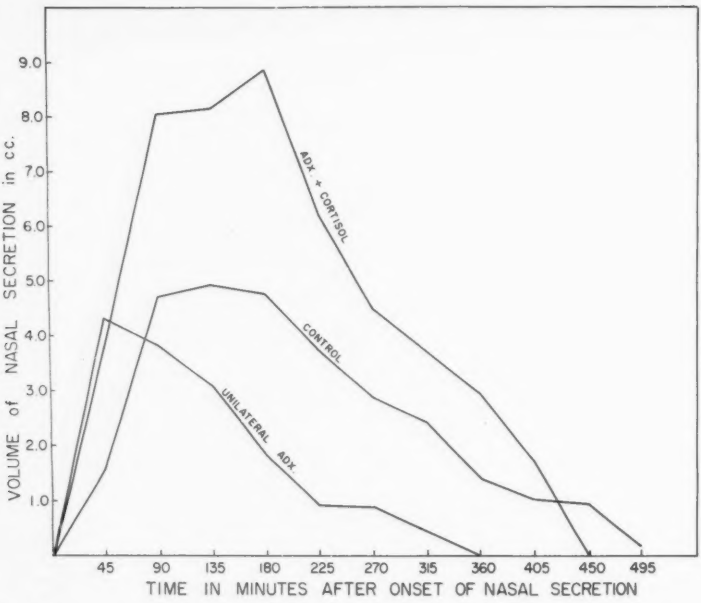

FIG. 1. The nasal secretion of intact, unilaterally adrenalectomized and totally adrenalectomized domestic ducks subsequent to saline loading.

values were significantly lower than the controls ($P < .05, < .01$ and $< .02$ respectively); however, the concentrations of sodium and potassium were not significantly different from the controls.

The totally adrenalectomized animals showed a complete inhibition of extra-renal activity in all but one bird. This bird showed a transient secretion, which could not be measured, 270 minutes after loading and at autopsy an adrenocortical remnant of approximately 5 mg. was found.

TABLE 2. THE VOLUME AND ELECTROLYTE COMPOSITION OF NASAL DISCHARGE FROM TOTALLY AND SUBTOTALLY ADRENALECTOMIZED DOMESTIC DUCKS FOLLOWING A SALINE LOAD

	Body weight, Kg.	Total Output				Concentration		
		Volume, ccs.	Na^+ m.eq.	K^+ m.eq.	$Na^+:K^+$ ratio	Na^+ m.eq./liter	K^+ m.eq./liter	$Na^+:K^+$ ratio
Intact Control	2.54 ±0.04	28.9 ±3.5	17.1 ±1.6	0.44 ±0.06	36.8 ±2.8	602.6 ±13.9	17.3 ±0.5	36.8 ±3.9
Unilateral ADX	2.44 ±0.05	15.5* ±3.9	8.6** ±2.2	0.21* ±0.05	32.2 ±7.4	571.3 ±25.0	14.4* ±1.2	40.4 ±2.2
Total ADX	2.38 ±0.04	Trace				Trace		
Total ADX + Cortisol	2.44 ±0.05	48.1 ±11.4	19.5 ±7.1	0.63 ±0.17	38.5 ±3.9	486.9** ±24.1	13.09** ±1.0	38.5 ±3.9

* $P < .05 > .01$ with respect to corresponding control value.
** $P < .01$ with respect to corresponding control value.

If the results from these adrenalectomized birds were considered in the most unfavourable manner with respect to the control birds then it could be stated that 1 out of 5 adrenalectomized birds showed a measurable nasal secretion whereas 10 out of 10 control birds showed measurable nasal secretions. By ascribing +ve or −ve values to the animals in each group, dependent upon whether or not they secreted, the Fisher exact probability test for nonparametric data could be applied (6). This analysis indicated a very high degree of significant difference between the adrenalectomized and control birds with respect to nasal secretion ($P < .001$).

Maintenance of the adrenalectomized birds with cortisol restored the extra-renal response and although the mean values for total volume and total sodium and potassium outputs are somewhat higher than the control values they are not significantly different ($P > .1$, $> .7$ and $> .3$ respec-

TABLE 3. THE COMPARISON OF SERUM SODIUM LEVELS IN INTACT AND UNILATERALLY AND TOTALLY ADRENALECTOMIZED DOMESTIC DUCKS AT THE ONSET OF NASAL SECRETION

Group	Time of onset of nasal secretion mins.	"P" value	Initial rise in serum Na^+ m.eq./liter/hr.	"P" value	Serum Na^+ at onset of nasal secretion me.q./liter	"P" value
Intact Control	52.8 ± 11.3	—	28.7 ± 2.3	—	160.6 ± 2.5	—
Unilateral ADX	116.6 ± 9.4	< .001	28.4 ± 4.8	> .9	170.2 ± 3.4	< .05 > .02
Total ADX	270*	—	32.8 ± 3.3	< .3 > .2	176.0	—
Total ADX + Cortisol	30.2 ± 14.8	< .3 > .2	28.7 ± 2.3†	—	155.7 ± 3.4†	< .3 > .2

* Only one bird of the group showed any nasal secretion.
† Intact control serum values used for these calculations.

tively). It is interesting to note, however, that the concentrations of sodium and potassium in the nasal secretion from these ducks were significantly lower than the concentrations observed in the intact controls and the unilaterally adrenalectomized birds ($P < .01$).

Serum changes: Table 3 and Figure 2

The pre-loading serum sodium and potassium values in the intact and unilaterally adrenalectomized ducks did not differ (144.1 ± 0.9 and 3.43 ± 0.07 m.eq./l. vs 145.6 ± 2.2 and 3.79 ± 0.17 m.eq./l. of sodium and potassium respectively) although birds which had been totally adrenalectomized for 12 hours showed a significantly lower value. The mean pre-loading serum sodium level for totally adrenalectomized birds (138.0 ± 1.7 m.eq./l.) was significantly lower than the corresponding control value ($P < .01$). The initial rise in serum sodium after saline loading did not

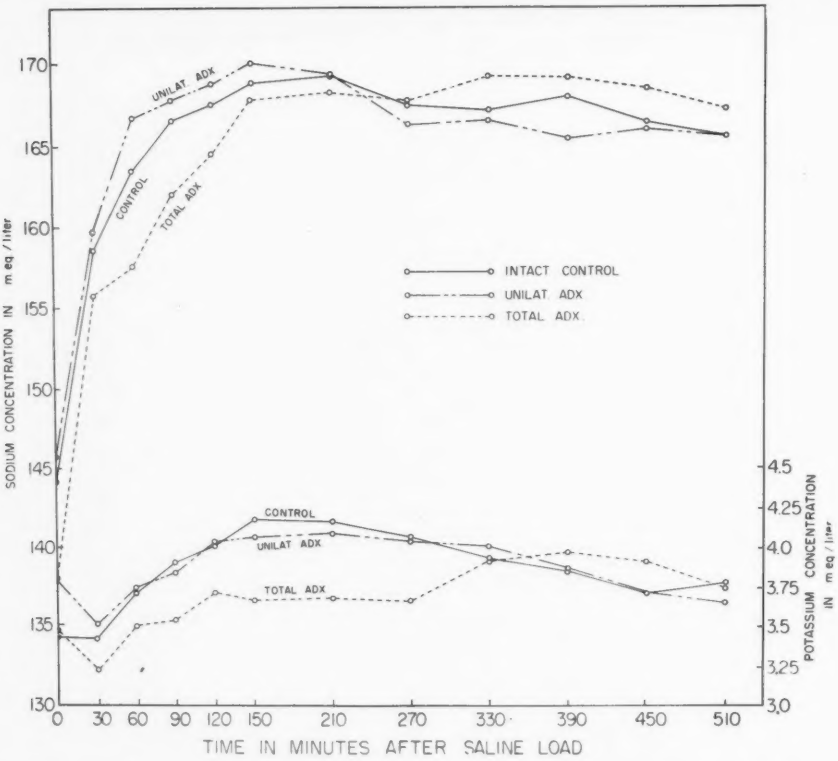

FIG. 2. The serum sodium and potassium changes in intact and adrenalectomized domestic ducks subsequent to saline loading.

differ in any of the groups; however, the totally adrenalectomized birds showed some lag in attaining the maximum serum sodium levels of the unilaterally adrenalectomized and intact controls.

The serum sodium concentration at the onset of nasal secretion in the unilaterally adrenalectomized birds was 170.2 ± 3.4 m.eq./l. and was significantly higher than the control value of 160.6 ± 2.5 m.eq./l. ($P < .05$). Although statistical analysis was not possible, the sodium level at onset in the one totally adrenalectomized bird which secreted was even higher (176.0 m.eq./l.).

Total adrenalectomy did not appear to affect the preloading level of potassium but the rise in serum potassium levels after loading did not occur to the same degree or so rapidly in this group as was observed in the controls and unilaterally adrenalectomized birds.

The time of onset of nasal secretion was considerably delayed in the unilaterally adrenalectomized birds (52.8 ± 11.3 min. vs 116.6 ± 9.4 min.). This delay was highly significant ($P < .001$). The maintenance of adrenal-

ectomized birds with cortisol reduced this time to 30.2 ± 14.8 minutes which was not significantly different from the control value ($P > .3$).

DISCUSSION

Recent investigations by Holmes, Phillips and Butler (5) have shown that in the normal duck given a hypertonic saline load both the amount and rate of secretion, principally of sodium chloride, from the nasal glands was significantly increased by the simultaneous administration of either cortisol, cortexone, aldosterone or mammalian ACTH. In this paper, it has been demonstrated that bilateral adrenalectomy in the duck abolished the ability of the nasal gland to respond to a saline load. This ability could be restored in the adrenalectomized duck by the administration of cortisol. It is clear, therefore, that one essential controlling factor of nasal secretion, namely the adrenocorticosteroids, must be added to those involving the nervous system (7, 8).

Birds which normally manifest the phenomenon of extra-renal secretion in response to a salt load can be made to secrete from the nasal glands by such other methods, as increasing the blood osmolarity through intravenous infusion of sucrose (3) or by the injection of certain adrenocorticosteroids without the addition of hypertonic fluids (5). The secretion achieved by the latter means was, however, only transient and it is concluded that the full expression of the response was dependent on the increased osmolarity of the blood together with the elevated levels of circulating adrenocorticosteroids. This introduces the concept that before the nasal gland will function fully the required threshold values must be exceeded not only for the degree of hypertonicity of the blood but also for the circulating amounts of corticosteroids. Moreover, it is clear that nasal secretion cannot occur in the adrenalectomized duck, however elevated might be the osmolarity of the extra-cellular fluid, whilst cortisol administration following adrenalectomy effectively re-establishes the extra-renal mechanism. Thus, the adrenocorticosteroids have a direct and essential action on the extra-renal salt glands. Further, the delay in onset, the duration and the magnitude of the extra-renal response was proportional to the level of circulating adrenocorticosteroids since unilaterally adrenalectomized ducks, exhibiting relative adrenocortical insufficiency, showed a delayed extra-renal response which occurred at a higher blood osmolarity than that seen for the control ducks. Conversely, simulated hyperadrenocorticalism (5) accelerated the onset of the extra-renal response but at a lower value for blood osmolarity. These contrasting conditions implied that a reciprocity existed between the concentration of circulating corticosteroids and the osmolarity of the extra-cellular fluid. This relationship was well illustrated in the one adrenalectomized animal which was found at autopsy to possess a small adrenal remnant, thus manifesting severe adrenal insufficiency. In this animal, a transient secretion from the nasal glands occurred after

a very long delay and with the highest concentration of serum sodium recorded in these and previous studies (5).

The day-to-day fluctuations in electrolyte intake of laboratory ducks and marine birds on similar fresh water diets are well within the capacity of the kidney alone to regulate the electrolyte and fluid distribution between the various compartments of the body (9). Under conditions of saline loading in the duck and in marine birds after the ingestion of sea water there is a dual and diphasic response (3, 5), the renal phase preceding and being of shorter duration than the extra-renal phase. Thus, in the normal physiology of a marine bird it would follow that the nasal gland only functions fully under conditions of hyperosmolarity with attendant hypersecretion of adrenocorticosteroids and under these conditions alone the normal capacity of the kidney to regulate is exceeded. It can be supposed, therefore, that the experimental addition of sodium chloride to ducks mimics a normal dilemma of the marine bird and brought into play the capacity to secrete, through the nasal glands, a saline solution which was three to four times more concentrated than the extra-cellular fluid, thereby maintaining homeostasis by reversing the trend to accumulate sodium and lose water from the body. Hence the change from renal to extra-renal routes of excretion in the saline loaded duck is a dynamic shift to a more economical method of restoring homeostasis.

The ducks investigated here and by Holmes, Phillips and Butler (5) showed a negative water balance in the initial period after the administration of a saline load, but later, the urine formation diminished and finally ceased. Thus, the renal phase waned as the extra-renal phase became dominant. Whilst undoubtedly the initial water loss may rest on the osmotic demands of the excretion of excess sodium both in control and adrenalectomized animals it would appear likely that hormones intervene in the determination of the pattern of the renal phase. One such hormonal factor may well be vasotocin, the antidiuretic hormone (ADH) known to occur in birds (10). In mammals saline administration evokes a secretion of ADH (11) and there is a relationship between the activities of the adrenocortical hormones and the antidiuretic hormone at the kidney level (12, 13). It can, therefore, be argued that the saline loading of control, adrenalectomized and adrenalectomized ducks receiving cortisol resulted in an increased ADH secretion with consequent water reabsorption and cessation of urine formation. Concomitantly, increasing amounts of sodium were reabsorbed with an increasing rate of adrenocorticosteroid secretion in the control ducks and the injection of cortisol into adrenalectomized ducks mimicked this condition. Thus, the pattern of renal excretion of salt and water in the control and cortisol injected adrenalectomized ducks was consistent with adrenocortical hypersecretion combined with enhanced ADH secretion since there was a gradual fall with time in sodium ouput, urine sodium concentration and volume of urine produced. On the other hand,

in the adrenalectomized ducks there was a marked polyuria with enhanced sodium loss. However, the diminished glomerular filtration rate in the absence of ACH together with the possible increasing secretion of ADH consequent on an elevated blood osmolarity, may have determined the reabsorption of water and so the termination of urine formation; although the time during which urination occurred was greatly protracted compared with control animals. Cortisol treatment of the adrenalectomized ducks given a saline load restored the renal phase to normal presumably by increasing the glomerular filtration rate, by its positive action as a diuretic agent, by enhancing the reabsorption of sodium in the kidney tubule and by restoring the secretory capacity of the extra-renal salt glands.

It might be argued that the differences between the normal and adrenalectomized ducks in their handling of a saline load by renal and extra-renal mechanisms could be accounted for, on the one hand, by increased corticosteroid secretion causing enhanced renal sodium retention and thereby elevating serum sodium values, whilst on the other, the absence of adrenocorticosteroids in the adrenalectomized animal resulted in renal sodium loss and a depression of the serum sodium values. However, observations revealing equivalent serum sodium values in both normal and adrenalectomized saline loaded animals, despite the significantly lower values in the latter prior to loading, vitiates this approach and emphasizes the essential role of the adrenocorticosteroids in determining the pattern of the di-phasic response.

The predominant adrenocortical secretions found in birds are corticosterone and aldosterone (14, 15, 16). If the secretory pattern is similar in the duck, then on general grounds, saline loading would be expected to inhibit aldosterone secretion as it does in the mammal (17). The nasal gland, however, will only respond fully when elevated levels of adrenocorticosteroids are present and it is therefore presumed that the glucocorticoid component of the adrenocortical secretion is responsible. The efficacy of cortisol in restoring the extra-renal phase to the adrenalectomized saline loaded duck substantiates this conclusion. Further, cortisol but not aldosterone is effective in causing nasal secretion to occur in the normal duck in the absence of a hypertonic saline load (9). The factor responsible for the elevated levels of adrenocorticosteroids may well be ACTH (5) and it is interesting in this connection that other workers (16) have found that the injection of ACTH into various gallinaceous birds significantly enhances the corticosterone levels found in the adrenal venous effluent.

It is very relevant to consider that if, in the saline loaded duck and the marine bird, ingestion of hypertonic fluids leads to an enhanced secretion of ADH, then this factor may well evoke an enhanced secretion of adrenocorticosteroids. It has been shown in mammals that saline loading stimulates adrenocortical activity (18) and the posterior lobe hormones are

clearly involved (19, 20). On this analogy we can say that ADH secretion either brings about an obligatory release of ACTH (18) or that ADH stimulates directly the biosynthetic pathways of adrenocorticosteroidogenesis (20). In the duck, then, one or both of these mechanisms may operate to effect an enhanced secretion of ACH, thereby overcoming a threshold value to cause extra-renal excretion of a fluid which is hypertonic to extra-cellular fluids. The combined effect of enhanced ADH and ACH secretion suppresses the kidney so that urine formation ceases. The extra-renal phase, however, persists under the influence of ACH until either the osmotic "stress" is removed or the circulating adrenocorticosteroids fall below the threshold level. In the marine bird it is envisaged that the following sequence of events occurs:—ingestion of sea water, elevation of the sodium chloride content of the blood, rise in the level of adrenocorticosteroids, nasal gland secretion, reduction of the sodium chloride content of the blood, fall in the level of circulating adrenocorticosteroids and cessation of nasal gland secretion.

In the present experiments when saline was given acutely, the nasal gland secretion waned and in some cases terminated despite high osmolarity of the blood. There may, then, have been excessive stimulation of adrenal tissue to a point when the enhanced biosynthesis of adrenocorticosteroids could no longer be maintained. It is known, for example, that adrenocortical exhaustion can be produced by excessive ACTH stimulation in the capon (14). Such a suggestion is supported by the observations on unilaterally adrenalectomized ducks, when the decrease in adrenocortical tissue and secretion is reflected by a marked shortening of the period during which extra-renal secretion occurs.

In conclusion we suggest that adrenocorticosteroids of the glucocorticoid type are essential for the functioning of the extra-renal salt glands in the duck and together with an antidiuretic hormone determines the characteristic diphasic response to hypertonic saline loads. These hormonal mechanisms together effectively restore homeostasis by causing a dynamic shift from the renal route to the physiologically more economical extra-renal route of excretion. The survival value of these mechanisms is self-evident and is especially advantageous to marine birds, who by virtue of their ecological niche are subjected to unusually high intakes of sea water or marine invertebrates.

The exact locus of action of the adrenal hormones on the nasal gland is not known and remains the subject for future investigations. The recent report of the Hokins (21) would, however, suggest an involvement in some stage of the phosphatidic acid cycle.

Acknowledgments

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BIOGENESIS OF ESTROGENS BY THE HUMAN OVARY:[1] FORMATION OF NETURAL STEROID INTERMEDIATES FROM PROGESTERONE-4-C^{14}, ANDROSTENEDIONE-4-C^{14} AND CHOLESTEROL-4-C^{14}

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ABSTRACT

The neutral steroids formed by gonadotropin-stimulated human ovaries incubated with C^{14} labeled progesterone, androstenedione and cholesterol have been subjected to various chemical procedures aimed towards their purification and identification. Criteria for radiochemical purity were provided for androstenedione, 17αhydroxyprogesterone and testosterone from progesterone, and for testosterone from androstenedione. On the basis of measurements by isotopic dilution, the comparative yields of the phenolic and neutral steroids from these and previously reported experiments of this series have been estimated. The qualitative and quantitative significance of these data are discussed.

THIS report concerns a continuation of *in vitro* studies aimed at evaluating both the quantitative and qualitative aspects of ovarian estrogen biosynthesis. By stimulating normal human ovaries with gonadotropin prior to their surgical removal, a biosynthetic system capable of a high level of estrogen production has been provided (1–4). An intermediary role of the Δ^4-3-ketosteroids in the ovarian synthesis of estrogen from acetate and cholesterol was first proposed by Solomon *et al.* in 1956 (5) on the basis of experimental evidence then available. Studies of the neutral steroids from the C^{14} labelled acetate experiment of the present series of experiments lent support to this proposal, since radioactive cholesterol, progesterone (pregn-4-ene-3,20-dione), 17αhydroxyprogesterone (17αhydroxypregn-4-ene-3,20-dione) and androstenedione (androst-4-ene-3,17-dione) were all identified (6) as well as estrone and estradiol 17β (1). Of particular interest, however, was the finding of radioactive pregnenolone (pregn-5-ene-3βol,20-one), 17αhydroxypregnenolone and dehydroepiandrosterone (androst-5-ene-3βol-17-one) (6). Although the conversion of progesterone to 17αhydroxyprogesterone and androstenedione by ovarian tissue had been previously

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demonstrated (5), and extraction of ovarian tissue had revealed the presence of endogenous androstenedione and 17αhydroxyprogesterone (7, 8), the possibility of a second pathway of ovarian estrogen biosynthesis, through the Δ^5-3βol neutral steroids, had not previously had any experimental basis. Furthermore, in none of the studies on ovarian neutral steroids (5–8) was testosterone (17βhydroxyandrost-4-en-3-one) identified. This has only recently been accomplished in studies with normal human ovarian tissue incubated with tritiated progesterone and C^{14} labelled 17αhydroxyprogesterone (9).

The present communication reports the findings in the neutral fractions from the C^{14} labeled progesterone, androstenedione and cholesterol experiments of this series, including the demonstration of the formation of testosterone from progesterone-4-C^{14} and from androstenedione-4-C^{14}. In addition, the quantitative relationships of the phenolic and neutral steroids isolated from 3 separate ovaries incubated with 4 different radioactive precursors have been tabulated and provide a basis for current concepts of the steps involved in estrogen formation.

MATERIAL AND METHODS

The ovaries for these experiments were obtained at surgery from three normally menstruating women who had been treated pre-operatively with gonadotropic preparations. In each case the follicular cyst linings from the enlarged, polycystic ovaries were either minced or homogenized in Krebs' phosphosaline buffer at pH 7.4 for incubation with a C^{14} labeled substrate. Details of the procedures used for tissue preparation and incubation have been previously presented in the reports on the estrogens isolated from these same experiments (1–4).

Mrs. B. (F.H.W. Path. #S-60-3070) had received 195 mg. of purified ovine Follicle Stimulating Hormone (F.S.H., National Institutes of Health) over a 9 day period just prior to surgery. The minced follicular cyst linings from one-half of one ovary ($\pm$2 gm. of tissue) were incubated with 2.12×10^6 c.p.m. of progesterone-4-C^{14} (30.7 μc/mg.; New England Nuclear Corp.) that had been previously purified on a Celite column.

Mrs. B.H. (F.H.W. Path. #S-61-00740) had received 950 mg. equivalents of Armour Standard F.S.H. (Armour Pharmaceutical Co.) over a 19-day period just before operation, plus 20,000 I.U. of human chorionic gonadotropin (Follutein, E.R. Squibb & Sons) during the last 4 days. An homogenate of the follicular cyst linings from both ovaries (11.2 gm. of tissue) was incubated with 1.46×10^6 c.p.m. of androstenedione-4-C^{14} (7.6 μc/mg., New England Nuclear Corp.) that had been previously purified on a Celite column.

Mrs. M.F. (F.H.W. Path. #S-60-3754) was treated with 175 mg. of ovine F.S.H. (National Institutes of Health) over a 9-day period just before laparotomy. The minced follicular cyst linings from both ovaries (1.97 gm. of tissue) were incubated with 1.46×10^6 c.p.m. of cholesterol-4-C^{14} (37.7 μc/mg., New England Nuclear Corp.). A control experiment with the same amount of this same preparation of C^{14} labeled cholesterol was run simultaneously throughout the processing of the incubated mixture, in order to eliminate the possibility of interferring contaminants.

The methods used for the extraction of the incubation mixture and fractionation of steroids, for the measurement of radioactivity and the quantitative assay of carriers, for Celite column chromatography, further purification by paper chromatography, and for

the formation of derivatives by both chemical and enzymatic conversion have been previously described (1–3, 6).

RESULTS

A. Neutral steroids from Progesterone-4-C^{14}.

Initial extraction and fractionation. The incubation mixture was extracted successively with small portions of hot ethanol to a total of 3 volumes and hot acetone to a total of 20 volumes. The alcohol-acetone-aqueous mixture, after filtration, was evaporated under reduced pressure to 5 ml. and extracted 3 times with 30 ml. of chloroform. The chloroform extract, containing 1.95×10^6 c.p.m. (92% of the starting material), was evaporated, partitioned between pentane and 90% methanol, and the methanol soluble material separated into "estriol," "estrone-estradiol" and "neutral" fractions as previously described (1). Total recovery at this point was 1.75×10^6 c.p.m. in the "neutral" fraction. Results on the phenolic fractions have already been reported (2). The neutral fraction was subjected to a Girard separation with the recovery of 70% of the radioactivity in the ketonic fraction and 1.1% in the non-ketonic. Low recovery was due to technical difficulties with emultions, resulting in loss of ketonic material.

Purification of ketonic neutral steroids on a Celite column. Figure 1. To the ketonic fraction (910,200 c.p.m.) the following carrier steroids were added; 200.8 μg of progesterone, 208.5 μg of androstenedione and 209.4 μg of 17α-hydroxyprogesterone. It was then applied to a 10×265 mm. Celite gradient elution column. After 99 tubes of one-tenth column volume each had been collected, a gradient of ethylene dichloride was applied. The last 30 tubes before the gradient showed only a gradual diminution of radioactivity to around 700 c.p.m. per tube, and have been omitted from Figure 1. The first six column volumes of gradient (tubes 101 through 113) were cut at one-half column volume per tube. Thereafter the cuts were made at one-tenth column volume. After 102 tubes of this gradient had been collected a second gradient of ethylene dichloride:methanol (1:1) was applied and another 45 tubes of one-half column volume each collected. This second gradient and the last 30 tubes of the first gradient contained only minor peaks of radioactivity and are not shown in Figure 1. Alternate tubes from the column were counted and in the areas of concentration of radioactivity every tube was counted and carrier steroids assayed by ultraviolet absorption. Of the applied radioactivity 98.5% was recovered. The recovery of the three carrier steroids was 97.5 to 100.7%. The tubes encompassing peaks of radioactivity were pooled, as indicated in Figure 1, for further processing. Those corresponding to the carrier steroids androstenedione and 17α-hydroxyprogesterone, pools 3 and 4 respectively, were further purified as will be described below. Pool 2, which corresponded to the carrier steroid progesterone, contained 25.6% of the radioactivity applied to the column and 11% of the progesterone-4-C^{14} added to the

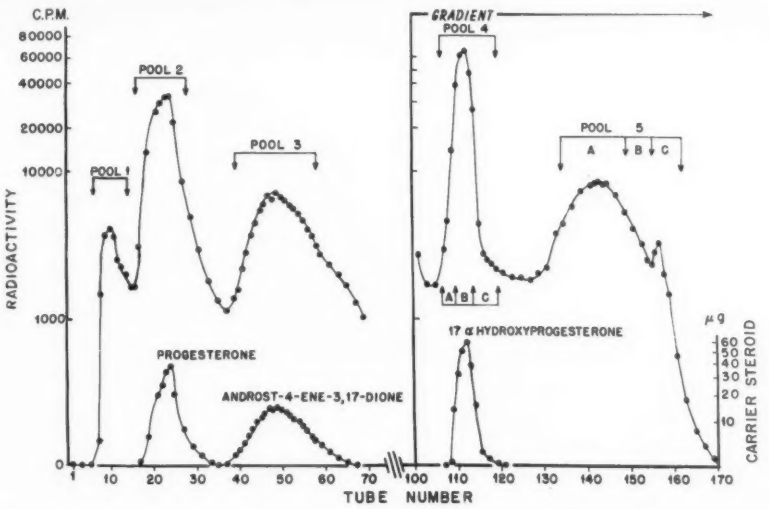

FIG. 1. *Celite gradient elution chromatography of the neutral, ketonic steroids from the incubation of a human ovary with progesterone-4-C^{14}.* The stationary phase was 90% methanol, the mobile phase was trimethylpentane and the gradient was ethylene dichloride. The cuts were made at one-tenth column volume per tube except for the first 13 tubes of the gradient, in each of which one-half column volume was collected. Distribution of carrier steroids and radioactivity is shown in the figure. Individual tubes were pooled as indicated and processed separately as described in the text.

tissue preparation for incubation. Pool 1, 2 and 5, which did not correspond to the carriers, have been set aside for future attempts at identification.

Purification of Androstenedione from Progesterone-4-C^{14}. Table 1. In the nine tubes from the androstenedione section of the Celite column that were taken for purification (Fig. 1, pool 3), the specific activity was 472 ± 11 c.p.m./μg with a chi-square of 2.03 and a p value of 0.99. This pool was run successively through three systems of paper chromatography, analyzing the eluted androstenedione sections for radioactivity and carrier. In each case the radioactivity ran with the carrier and there was little change in specific activity. The eluted androstenedione section from the third paper chromatograph was incubated with a placental enzyme system which converts androstenedione to estrone with a high degree of specificity (10). The incubation mixture was extracted with chloroform and the estrogen metabolites purified by phenolic separation, methylation, hydrogen peroxide treatment with NaOH:petroleum ether partition, and chromatography on an alumina column. These steps involved a considerable loss of radioactivity, but the estrone methyl ether fraction[2] from the alumina column had the same specific activity as that of the starting androstenedione, thus

[2] All values for conjugated steroids are expressed in terms of the free compounds.

TABLE 1. PURIFICATION OF RADIOACTIVE ANDROSTENEDIONE FROM PROGESTERONE-4-C^{14}, AND CONVERSION OF THE ANDROSTENEDIONE TO ESTRONE

Purification steps (successive)		Radio-activity, c.p.m.	Carrier steroid, μg	Specific activity, c.p.m./μg
Celite column (9 tubes)		55,800		472 ± 11†
Paper chromatography				
Ligroin:Toluene (2:1)/70% methanol		41,300	86.9	475
Ligroin/96% methanol		29,150	70.1	416
Ligroin/Propylene glycol		23,100	53.2	434
Incubated with placental enzyme, extract assayed for estrone				
Crude extract		15,650	*	
Phenolic fraction		12,850	*	
Methylation and NaOH:petroleum ether partition		6,760	*	
Alumina chromatography		4,960	10.6	468
Carrier estrone methyl ether added		c.p.m.	mg	c.p.m./mg
Pool assay		4,200	9.00	467
1st crystallization	crystals	3,500	7.60	461
	mother liquor	700	1.50	467
2nd crystallization	crystals	2,900	6.25	464
	mother liquor	650	1.42	458

* Fluorescence could not be measured because of quenching phenomenon.
† Ave. ±S.D. of 9 tubes that made up the pool. The c.p.m. recorded = sum of tube assays.

providing additional evidence for radiochemical identity. Recrystallized estrone methyl ether (m.p. 171–172° C) was added to the estrone fraction from the alumina column and two successive crystallizations from benzene: ethanol carried out, assaying both the crystals and mother liquor for radioactivity and fluorescence. The results revealed a constant specific activity which did not differ from the specific activity of the 9 tubes pooled for purification from the androstenedione section from the Celite column (pool 3, Fig. 1).

It seems safe to assume, therefore, that the 55,800 c.p.m. of radioactivity of this pool represented essentially pure androstenedione. If this figure is corrected for the 29% loss of ketonic material in the Girard separation before applying the extract to the Celite column (v.s.), the amount of androstenedione formed from the incubated progesterone-4-C^{14} may be estimated at approximately 78,000 c.p.m., or 3.7% of the starting material.

Purification of 17αhydroxyprogesterone from Progesterone-4-C^{14}. Table 2. The 17αhydroxyprogesterone section from the Celite column was divided into 3 parts in order to take advantage of any slight separation of 17αhydroxyprogesterone from testosterone, if present. The 4 peak tubes of this section (pool 4-B, Fig. 1) were pooled for purification of 17αhydroxyprogesterone. The specific activity of the pool assay agreed with that of the peak tube. After one paper purification the eluted 17αhydroxyprogesterone section was acetylated and run on another system of paper chromatog-

raphy against a testosterone acetate standard. The bulk of the radioactivity (68% of the total) remained in the 17α-hydroxyprogesterone section, but a second concentration of counts (less than 0.1% of the total) was observed in the testosterone acetate section and was set aside for further study (see below). A third paper purification of the eluted 17α-hydroxyprogesterone section gave good recovery of radioactivity with no change in specific activity. The pool was next subjected to chromic acid oxidation and run on a paper chromatograph against an androstenedione standard. Conversion to a compound of the same polarity as androstenedione, again with no change in specific activity, provided further evidence for its radiochemical identity. The eluted androstenedione section, when incubated with a placental enzyme system that converts androstenedione to estrone, was analyzed for estrone before and after purification by methylation and column chromatography; again with no change in specific activity from that of the "peak" 17α-hydroxyprogesterone pool from the Celite column.

It may be assumed, therefore, that the 220,000 c.p.m. of pool 4-B (which amounted to 78% of the total 17α-hydroxyprogesterone section) represented essentially pure 17α-hydroxyprogesterone. On this basis, and correcting for the 29% loss of ketonic material in the initial processing, the amount of 17α-hydroxyprogesterone formed from the incubated progesterone-4-C^{14} may be estimated at approximately 314,000 c.p.m., or 14.8% of the starting material.

Purification of Testosterone from Progesterone-4-C^{14}. Table 3. Each of the 3

TABLE 2. PURIFICATION OF RADIOACTIVE 17α-HYDROXYPROGESTERONE FROM PROGESTERONE-4-C^{14}, OXIDATION OF THE 17 HYDROXYPROGESTERONE TO ANDROSTENEDIONE, AND CONVERSION OF THE ANDROSTENEDIONE TO ESTRONE

Purification steps (successive)	Radioactivity, c.p.m.	Carrier steroid, μg	Specific activity, c.p.m./μg
Celite column (4 tubes)	220,000		
Peak tube	68,340	57.5	1188
Pool assay	215,500	173	1246
Paper chromatography			
Ligroin:Toluene (2:1)/70% methanol	169,400	152	1114
Acetylation and paper chromatography			
Ligroin/96% methanol	109,200	*	
Toluene/75% methanol	100,100	83.4	1200
CrO_3 oxidation, analysis for androstenedione Paper chromatography			
Ligroin/96% methanol	66,160	53.5	1237
Incubated with placental enzyme, extract assayed for estrone			
Phenolic fraction	44,720	35.2	1270
Methylation and NaOH:petroleum ether partition	38,180	*	
Alumina chromatography	33,600	26.6	1263

* Contaminant interfered with assay of carrier.

pools from the 17α-hydroxyprogesterone section from the Celite columns (pools 4-A, B and C, Fig. 1) were acetylated separately and run on paper chromatographs against 17α-hydroxyprogesterone and testosterone acetate standards. Sections corresponding to the standards and equidistant sections above and below were cut for elution and assay. In the "peak" pool (4-B), as reported above, less than 0.1% of the applied radioactivity was concentrated in the testosterone acetate section. This, however, when rechromatographed in the same system of paper chromatography, gave 58% recovery in the testosterone acetate section and no other concentration of counts on the paper. In pool 4-C, the more polar section from the Celite column, there was no concentration of counts that corresponded to testosterone acetate, the only peak of radioactivity being in the section which contained the unchanged 17α-hydroxyprogesterone carrier. In pool 4-A, however, a marked concentration of radioactivity appeared in the testosterone acetate section amounting to over 10% of the radioactivity applied. The only other concentration of counts was in the 17α-hydroxyprogesterone section where 42% of the radioactivity applied to the paper was recovered.

The eluted testosterone acetate sections from the paper chromatograms of pools 4-A and B(3310 c.p.m.) were combined and testosterone acetate carrier added.[2] After incubation with a placental enzyme system known to hydrolyze testosterone acetate[3] and to convert testosterone to estrone with a high degree of specificity (10), the phenolic fraction of the incubation mixture was purified, as described above, and assayed for estrone in the form of estrone methyl ether. There was a 26% drop in specific activity when the phenolic fraction was assayed, but no further change throughout these purification procedures. Recrystallized estrone methyl ether (m.p. 171–172° C) was added and three successive crystallizations from benzene: ethanol carried out, assaying both crystals and mother liquor for fluorescence and radioactivity. The specific activity of the three batches of crystals and of the third mother liquor did not differ from that of the pool assay before crystallization.

After the initial addition of carrier to the 3310 c.p.m. of the original testosterone acetate pool, therefore, there was a total change in specific activity indicating that 74% of this, or 2450 c.p.m., represented essentially pure testosterone.[2] Known losses prior to the addition of carrier would correct this figure to 3750 c.p.m., indicating that approximately 0.2% of the progesterone-4-C^{14} was converted to testosterone by incubation with ovarian tissue.

B. Neutral Steroids from Androstenedione-4-C^{14}

Initial extraction and fractionation. The incubation mixture was extracted 3 times with 6 volumes of chloroform and the residue (tissue and

[3] Unpublished observations.

TABLE 3. PURIFICATION OF RADIOACTIVE TESTOSTERONE FROM PROGESTERONE-4-C^{14}, AND CONVERSION OF THE TESTOSTERONE TO ESTRONE

Purification steps (successive)	Radio-activity, c.p.m.	Carrier steroid, μg	Specific activity, c.p.m./μg
Celite column (17 hydroxyprogesterone section)			
Pool 4 A (3 tubes)	21,900		
Pool 4 B (4 tubes)	215,500		
Pool 4 C (6 tubes)	40,430		
Acetylation and paper chromatography; analysis for Testosterone acetate			
Ligroin/96% methanol			
Pool 4 A	2,410		
Pool 4 B	1,630		
Pool 4 C	0		
A and B from above combined and carrier Testosterone Acetate added			
Pool assay	3,310	86.8	38.1
Incubated with placental enzyme, extract assayed for estrone			
Phenolic fraction	1,700	60.8	28.0
Methylation and NaOH-petroleum ether partition	1,470	52.7	27.9
Alumina chromatography	1,140	40.6	28.1
Carrier estrone methyl ether added	c.p.m.	mg	c.p.m./mg
Pool assay	950	4.67	203
Crystallization			
1st crystals	830	4.00	207
2nd crystals	730	3.58	204
3rd crystals	410	1.90	216
1st mother liquor	150	0.62	242
2nd mother liquor	91	0.35	260
3rd mother liquor	335	1.60	209

aqueous phase) treated with 20 volumes of hot acetone. After filtration the acetone-aqueous mixture was reduced to 5.0 ml. by evaporation in vacuo and extracted 3 times with 30 ml. chloroform. The combined chloroform extracts, containing 1.20×10^6 c.p.m. or 82% of the starting material, were evaporated to dryness, the residue partitioned between pentane and 90% methanol and the methanol soluble material separated into "estriol," "estrone-estradiol" and "neutral" fractions as previously described (1). Recovery at this point was 1.18×10^6 c.p.m., or 80.5% of the starting radioactivity, with 72% of this in the "neutral" fraction. Results on the phenolic fractions have already been reported (4).

Purification of neutral steroids on a Celite column. Figure 2. To the neutral fraction (847,500 c.p.m.) were added 216.7 μg of carrier androstenedione and 228.0 μg of carrier testosterone. It was then applied to a 10×266 mm. Celite gradient elution column. This was eluted and analyzed for radioactivity and carriers as described above for the initial chromatographic purification on a Celite column of the ketonic neutral fraction from the progesterone-4-C^{14} experiment (Section A). The fractions were cut at one

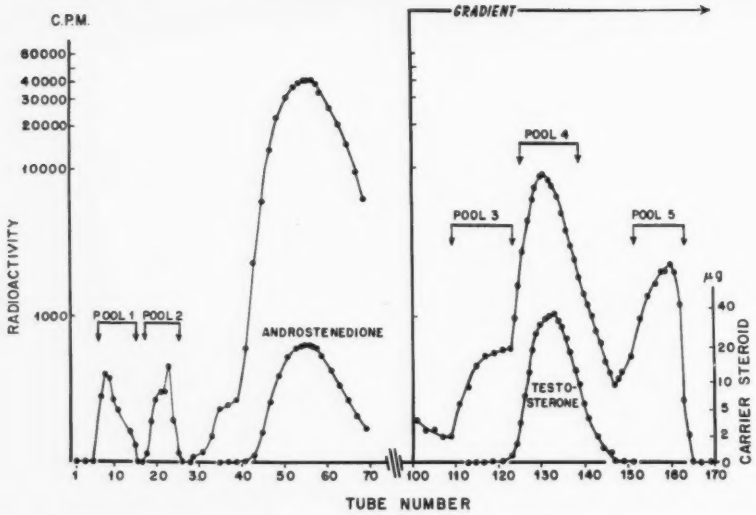

FIG. 2. *Celite gradient elution chromatography of the neutral steroids from the incubation of a human ovary with androstenedione-4-C*^{14}. The stationary phase was 90% methanol, the mobile phase was trimethylpentane and the gradient was ethylene dichloride. One-tenth of a column volume was collected per tube throughout. Distribution of carrier steroids and radioactivity is shown in the figure. Individual tubes were pooled as indicated and processed separately as described in the text.

tenth column volume per tube from the start of the chromatograph throughout the collection of the first gradient. The last 30 tubes before the first gradient showed nothing but a gradual diminution of radioactivity from the androstenedione peak to around 200 c.p.m. per tube, and have been omitted from Figure 2. The last 30 tubes of the first gradient and the 36 half column volume tubes of the second gradient are also omitted since they showed only minor peaks of radioactivity. Ninety percent of the radioactivity applied to the column was recovered before stripping it with methanol. Analyses for the carrier steroids by ultraviolet absorption showed smooth curves but gross overestimates as evidenced by the apparent recovery of considerably more of each carrier than had been applied. This difficulty, not encountered in any other analyses for Δ^4-3-ketones off of Celite columns, was probably due to the fact that this was the only experiment in which the whole neutral fraction (without partial purification by further fractionation) had been applied to the column.

Five pools were made up of tubes that corresponded to peaks of radioactivity; as shown in Figure 2. Tubes 45 through 69, which corresponded to the carrier steroid androstenedione, contained 70% of the radioactivity applied to the column and 40.5% of the androstenedione-4-C^{14} starting material. These tubes were not saved. Pools 1, 2 and 5, which were not

associated with the recovery of carrier steroids, were set aside for future investigation. The most interesting of these is pool 5 which appears to represent a major metabolite of androstenedione-4-C^{14}. In its polarity off the Celite column, it corresponds to pool 5 of the Celite column from incubated progesterone-4-C^{14} (Fig. 1), and also to an unidentified major metabolite of acetate-1-C^{14} which appeared in the ketonic, non-digitonide, neutral fraction (6).

Pool 3 represented some radioactive metabolite slightly less polar than testosterone which was not separated from the "testosterone" peak. Since large amounts of radioactive estrone had been recovered and isolated from the phenolic fraction of this incubation mixture (4) it was thought that some estrone, the polarity of which is close to that of testosterone, might have been left in the neutral fraction after phenolic separation. A repeat phenolic separation on pool 3, however, gave 90% recovery of its radioactivity in the neutral fraction, thus eliminating the possibility of any significant contamination, in this section of the column, with radioactive estrone. The neutral fraction of pool 3 (4660 c.p.m.) was set aside for future study.

Purification of Testosterone from Androstenedione-4-C^{14}. Table 4. Pool 4, Fig. 2, which contained 77,200 c.p.m. or 9.1% of the radioactivity applied to the column, corresponded to the carrier steroid testosterone, and the curves for radioactivity and ultra violet absorption were practically superimposable in the peak region. Because of the possibility of contamination with radioactive estrone, this pool was first put through 2 successive partitions between benzene:petroleum ether and NaOH to remove phenolic material. This involved some slight loss of radioactivity but 97.5% of the counts recovered from the second partition were in the neutral fraction. The next purification step was acetylation followed by paper chromatography. This whole procedure was done in duplicate using one-half of the pool for each run, in order to get as complete acetylation and recovery as possible of the testosterone carrier. The duplicates, for both radioactivity and carrier, checked very closely and the specific activities checked almost exactly. The total recovery of counts in the testosterone acetate sections from the paper amounted to 66% of the radioactivity before acetylation, showing that the bulk of the radioactivity ran with the carrier in both of these procedures. The 36,300 c.p.m. in the combined eluates from the papers were next incubated in one flask with a placental enzyme preparation known to hydrolyze testosterone acetate[3] and convert it to estrone (10). The chloroform extract of the incubation mixture was carried through a phenolic separation, methylation, purification of the methyl ether and column chromatography as described above (Section A), analyzing for radioactivity and estrone at each step. There was a 23% drop in specific activity, indicating purification of the radioactive metabolite, but the bulk of the radioactivity accompanied estrone, thus providing further evidence

TABLE 4. PURIFICATION OF RADIOACTIVE TESTOSTERONE FROM ANDROSTENEDIONE-4-C^{14} AND CONVERSION OF THE TESTOSTERONE TO ESTRONE

Purification steps (successive)	Radio-activity, c.p.m.	Carrier steroid, μg	Specific activity, c.p.m./μg
Celite column (14 tubes)	77,200	*	430†
Partition between 0.4 N NaOH and Benzene:petroleum ether (1:1)			
Benzene:petroleum ether #1	60,100	*	
Repeat above partition			
Benzene:petroleum ether #2	54,970	*	
Acetylation and paper chromatography			
Methylcyclohexane/propylene glycol on 2 strips			
Strip #1	17,670	57.5	307
Strip #2	18,600	61.0	305
Incubated with placental enzyme, extract assayed for estrone			
Phenolic fraction	18,700	66.4	282
Methylation and NaOH:petroleum ether partition	15,560	62.8	248
Alumina column chromatography	14,430	61.2	236
Carrier estrone methyl ether added	c.p.m.	mg	c.p.m./mg
Pool assay	13,200	5.78	2283
1st crystallization crystals	10,990	5.20	2114
1st crystallization mother liquor	790	0.239	3305
2nd crystallization crystals	4,310	1.90	2268
2nd crystallization mother liquor	6,990	3.10	2255

* The neutral fraction applied to the Celite column for this experiment had not been purified, as in previous studies of neutral fractions, by Girard separation or digitonin precipitation. As a result, the assays for the carriers by ultraviolet absorption gave gross overestimates. This difficulty was not resolved until after acetylation and paper chromatography of the testosterone pool.

† Estimated: see text.

for radio-chemical identity of the starting material with testosterone. To the estrone methyl ether fraction from the alumina column, carrier estrone methyl ether (m.p. 171–172° C) was added and two crystallizations carried out, analyzing both crystals and mother liquor for radioactivity and fluorescence. Constant specific activity was demonstrated for the pool, both batches of crystals and the second mother liquor, indicating that the radioactive estrone methyl ether, after column chromatography, was essentially pure.

The specific activity of pool 4 (Fig. 2), that was taken from the Celite column for purification and identification of testosterone, is unknown since some contaminant gave gross overestimates in the ultraviolet analyses. In order to obtain a base line for purposes of comparison, 80% (180 μg) of the carrier testosterone initially added was arbitrarily used in computing the reference specific activity of 430 c.p.m. for the pool prior to the purification steps shown in Table 4. Since the final specific activity by the time radio-

TABLE 5. COMPARATIVE YIELDS OF RADIOACTIVE METABOLITES AS RELATED TO SUBSTRATE

Radioactive metabolite	Substrate			
	Acetate-1-C^{14} 222×10^6 c.p.m.	Cholesterol-4-C^{14} 1.46×10^6 c.p.m.	Progesterone-4-C^{14} 2.12×10^6 c.p.m.	Androstenedione-4-C^{14} 1.46×10^6 c.p.m.
Cholesterol	0.34%			
Pregnenolone	0.0025%			
17 hydroxypregnenolone	0.0006%	*		
Dehydroepiandrosterone	0.0046%	*		
Progesterone	0.0002%			
17 hydroxyprogesterone	0.0013%	*	14.8%	
Androstenedione	0.0047%		3.7%	
Testosterone	*		0.2%	2.9%
Estrone	0.012%	0.09%	3.5%	10.9%
Estradiol	0.016%		2.1%	4.4%
Total estrogen	0.03%	0.09%	5.6%	15.3%

* Possibly a trace; not identified.

chemical purity had been achieved was 55% of this, we may estimate that the 77,200 c.p.m. of pool 4 from the Celite column represented about 42,500 c.p.m. of essentially pure testosterone. This would indicate a 2.9% conversion of androstenedione-4-C^{14} to radioactive testosterone by incubation with ovarian tissue.

Neutral Steroids from Cholesterol-4-C^{14}. Studies on the neutral steroids from the cholesterol-4-C^{14} experiment were handicapped by insufficient radioactivity in the starting material. The incubation mixture was extracted and fractionated as previously described and the ketonic neutral material separated into a digitonide and a non-digitonide fraction, each of which was run on a Celite column. The digitonide fraction (7100 c.p.m.) was processed with the added carrier steroids pregnenolone, dehydroepiandrosterone and 17αhydroxypregnenolone, and the non-digitonide fraction (12,000 c.p.m.) with the added carrier steroids progesterone, androstenedione and 17αhydroxyprogesterone. In the tube assays from the columns and in subsequent purifications, by successive paper chromatographs and the formation of derivatives, peaks of radioactivity accompanied the carrier steroids dehydroepiandrosterone, 17αhydroxypregnenolone and 17αhydroxyprogesterone. Trace amounts of these 3 metabolites, less than 0.02% of the starting radioactivity may have been formed, but positive identification was not possible.

DISCUSSION

For the overall consideration of the findings in the phenolic and neutral fractions from this series of studies with human ovaries, the estimated yield of each identified radioactive product has been calculated, and is shown in Table 5. The comparative yields of total estrogen are in keeping with current concepts concerning the biosynthetic pathway of estrogen formation, with ascending yields as the substrate approaches the end product, from acetate to cholesterol to progesterone to androstenedione to estrone and estradiol.

A number of factors could influence the accumulation of the neutral intermediate metabolites, and any interpretation of their comparative yields must be speculative. In the acetate-1-C^{14} experiment the total yields of Δ^5 3βol and Δ^4 3-ketosteroids are about the same, suggesting that, from a quantitative point of view, either or both of these pathways could be operative. The relatively high yield of cholesterol from acetate-1-C^{14}, as compared to the C_{21} and C_{19} metabolites, would suggest a rate limiting reaction in the conversion of cholesterol to pregnenolone. Once the C_{19} steroids have been formed, however, aromatization would appear to take place very readily, since the recovery of estrogens is relatively high. It is not surprising, on this basis, that in the cholesterol experiment, in which the starting radioactivity was of the same order of magnitude as that in the progesterone and androstenedione experiments, it was impossible to identify the neutral intermediates although positive identification of estrone was accomplished (3).

The identification of testosterone from progesterone and androstenedione is of particular interest and confirms the recent report by Kase *et al.* concerning the isolation of testosterone as well as androstenedione from progesterone and 17αhydroxyprogesterone (9). The identification of testosterone as a metabolite of three different neutral steroids, progesterone, 17αhydroxyprogesterone and androstenedione, all three of which have been isolated as endogenous constituents of ovarian tissue (7, 8), lends greater significance to testosterone as an ovarian estrogen precursor than was heretofore possible.

Our yields of androstenedione and of testosterone from progesterone are of the same order of magnitude as those reported by Kase *et al.*, with evidence for a greater accumulation of androstenedione than of the more biologically active androgen, testosterone. The recovery of androstenedione as a major metabolite of testosterone incubated with a variety of human tissues has been previously reported (11). Throughout these experiments, as has been observed in placental enzyme studies (10), the recovery of estrone has also been either greater than or equivalent to that of estradiol. Accumulation of the oxidized forms, androstenedione, and estrone, rather than of the more biologically active reduced forms, testosterone and estradiol, may depend upon the equilibria of the dehydrogenase reactions under the *in vitro* experimental conditions. The observed ratios of androstenedione to testosterone and of estrone to estradiol in the present studies could be a consequence of the method and not reflect ovarian secretion as it occurs *in vivo*. The 17βol dehydrogenase reaction involved in these interconversions is readily reversible, a characteristic that differentiates it from the Δ^4–Δ^5 isomerase reaction, the 17αhydroxylation, the cleavage of the side chain, and the aromatization steps in the biosynthetic pathway. The equilibrium of this reversible dehydrogenase reaction might well be easily influenced by its environmental conditions, both *in vitro* and *in vivo*.

Whatever the interpretation of the present data, they represent certain quantitative and qualitative potentials of the ovarian system for estrogen formation, and provide a basis for further detailed studies concerning the mechanisms involved.

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NOTES AND COMMENTS

THE INFLUENCE OF DIPPING EGGS IN MALE HORMONE SOLUTIONS ON LYMPHATIC TISSUE AND ANTIBODY RESPONSE OF CHICKENS[1,2]

ABSTRACT

Fertile eggs were removed from the incubator after three days of incubation and dipped in male hormone solutions. The bursa of Fabricius, a lympho-epithelial gland, was markedly reduced or eliminated by exposure to testosterone propionate. The antibody response to *Salmonella pullorum* antigen of 5-week-old birds hatched from testosterone propionate-dipped eggs was eliminated.

INTRODUCTION

The present study was undertaken to determine whether two glands closely related to lymphatic tissue, the thymus and bursa of Fabricius, would react the same when exposed to male hormone during their embryonic development. While the function of the thymus in chickens is not known, it now appears that the bursa of Fabricius, a lympho-epithelial gland, plays a major role in the antibody potential of the chicken (1–3).

MATERIALS AND METHODS

Fertile eggs were dipped in hormonal solutions on the third day of incubation. The treatment solutions were: (A) 2 gm. of testosterone propionate dissolved in 100 ml. of ethyl alcohol; (B) 670 mg. of testosterone propionate dissolved in 100 ml. of ethyl alcohol; (C) 670 mg. of dehydroisoandrosterone dissolved in 1 ml. of ethyl alcohol; and (D) ethyl alcohol. The pointed end of the egg was dipped for 5 seconds to a depth of 1½ inches (4). The temperatures of solutions and eggs were approximately 10° C and 37.5° C, respectively. At hatching, 12 chicks from each group were killed and at 2 weeks of age, 8 chicks from each group were killed. The weights of the body, bursa, and thymus were recorded. Differences between body weight means and gland weight means were determined by the analysis of variance and analysis of covariance, respectively (5). At 5 weeks of age, 7 birds hatched from the alcohol group and 7 birds hatched from eggs dipped in 2 gm. of testosterone were intravenously injected with 1 ml. of a polyvalent K *Salmonella pullorum* antigen. One week later, the birds were bled by cardiac puncture. The plasma was collected and an antibody titer determined for each bird (6). All tubes were read 6 hours after removal from the water bath.

RESULTS AND DISCUSSION

The body weight of 2-week-old chicks hatched from eggs dipped in 2 gm. of testosterone propionate was significantly lower than the body weight of birds hatched from eggs dipped in ethyl alcohol (Table 1). The mean weight of the bursae of chicks hatched from eggs dipped in male hormone solutions was significantly reduced at hatching and 2 weeks of age (Table 1).

Dipping eggs in 2 gm. of testosterone propionate resulted in the elimination of the bursa of Fabricius in 8 of 12 chicks at hatching and in 5 of 8 chicks at 2 weeks of age. Dipping eggs in 670 mg. of testosterone propionate resulted in the elimination of the bursa in 1 of 12 chicks at hatching and in 1 of 8 chicks at 2 weeks of age. Dipping fertile eggs in male hormone solutions did not significantly influence the weight of the thymus at hatching or 2 weeks of age (Table 1). The antibody titer of all 7 of the chicks hatched from eggs dipped in 2 gm. of testosterone propionate was 0. On the other hand, the antibody titer of 5 of the birds hatched from eggs dipped in ethyl alcohol was 1:64 while the remaining 2 birds showed an antibody titer of 1:128.

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[2] Journal article no. 937.

TABLE 1. THE MEAN WEIGHTS OF THE BODY, BURSA OF FABRICIUS, AND THYMUS OF CHICKS HATCHED FROM EGGS DIPPED IN MALE HORMONE SOLUTIONS AT THREE DAYS OF INCUBATION

	Body weight, gm.		Bursa of Fabricius,[1] mg.		Thymus, mg.	
	A[2]	B[2]	A[2]	B[2]	A[2]	B[2]
Ethyl alcohol	44 ± 4	148 ± 20	46 ± 12	372 ± 110	88 ± 22	678 ± 200
Dehydroisoandrosterone, 670 mg./100 ml. ethyl alcohol	44 ± 4	148 ± 20	40* ± 16	249* ± 150	88 ± 20	846 ± 300
Testosterone propionate, 670 mg./100 ml. ethyl alcohol	44 ± 3	145 ± 28	13** ± 8	76* ± 75	85 ± 17	662 ± 222
Testosterone propionate, 2 gm./100 ml. ethyl alcohol	46 ± 3	122* ± 24	3.5** ± 3.5	3* ± 3	85 ± 17	502 ± 225

A—Weighed at hatching.
B—Weighed at 2 weeks of age.
[1] The testosterone means include all the birds, with or without a bursa.
[2] All means were compared with the ethyl alcohol mean.
** $P \leq .01$.
* $P \leq .05$.

Under the conditions of our test, the thymus and bursa of Fabricius did not respond similarly to male hormone exposures. A third lymphatic gland, the spleen, was reported by Glick and Sadler (7) to be reduced in size but not eliminated after embryonic exposure to testosterone propionate. Therefore, the bursa of Fabricius appears to be distinctive in its sensitivity of response to testosterone. The elimination of the antibody response in 5-week-old birds hatched from testosterone propionate dipped eggs confirms our earlier report (7). It now appears that the elimination of the antibody response in these chicks can be primarily attributed to the lack of a functioning bursa.

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PREVENTION OF CALCIPHYLAXIS BY HYPOPHYSECTOMY[1]

ABSTRACT

In the rat, hypophysectomy, unlike adrenalectomy, prevents the intense cutaneous calcinosis normally induced by certain challenging agents administered during a "critical period" following systemic dihydrotachysterol (DHT) treatment.

In animals sensitized by pretreatment with parathyroid hormone, dihydrotachysterol (DHT) or other calcification-promoting agents, there develops a state of altered tissue reactivity for which the term "calciphylaxis" has been proposed. This condition is manifest only during a brief "critical period" following the systemic administration of the calcifying agent. It is characterized by the precipitous attraction of calcium (frequently conducive to sclerosis or necrosis) to regions treated with agents that appear to act as "vital mordants" for calcium (1).

There exists no parallelism between the local damage (inflammation, necrosis) caused by such mordants and their calcium-attracting power. For instance, many strong irritants (croton oil, formalin) and corrosives (NaOH, HCl, proteases) are ineffective, while mild stimuli (pinching of the skin, very dilute solutions of egg white or iron salts) can cause massive calcification with necrosis during the calciphylactic state. We also found no close correlation between the usual metastatic calcification (e.g., in the cardiovascular system or kidney) produced by a systemically applied calcifier and the ability of the same agent to induce a calciphylactic response to topically applied vital mordants. For example, chronic heavy overdosage with parathyroid hormone or DHT can cause widespread metastatic calcification without inducing the characteristic local response to mild irritation, while during the "critical period," precipitous calciphylactic reactions can be produced without any noteworthy calcium deposition outside of the challenged area. Thus, even mere plucking of the hair or subcutaneous injection of as little as 25 μg of $FeCl_3$ (either of which normally produces no detectable sign of tissue injury) causes necrotizing cutaneous calcinosis, but only on the second or third day after the DHT administration.

A great variety of lesions (e.g., pancreatic, splenic, uterine, hepatic, biliary, duodenal or salivary gland calcification with subsequent necrosis or sclerosis) can thus be produced rather selectively during the calciphylactic state by the intravenous or intraperitoneal administration of appropriate challenging agents that exhibit a particular affinity for certain target organs. These observations have been described in detail elsewhere (1, 2, 3); here, we shall report only upon experiments indicating that hypophysectomy completely protects against certain forms of this type of abnormal reactivity.

MATERIALS AND METHODS

Fifty female Holtzman rats with a mean initial body weight of 100 gm. (range 95–106 gm.) were subdivided into five equal groups as indicated in Table 1. *Adrenalectomies* (costovertebral approach) and *hypophysectomies* (parapharyngeal approach) were performed under ether anesthesia on the first day. Preliminary observations having shown that glucocorticoids do not prevent calciphylaxis, *triamcinolone* (100 μg, in 0.2 cc. of water subcutaneously, once daily) was given—for life maintenance—to the adrenalectomized and hypophysectomized animals, as well as to one group of intact controls. *DHT* (dihydrotachysterol, Calcamin®, Wander) was administered at the dose of 1 mg. in 0.5 cc. of corn oil, by stomach tube, once, on the third day. We allowed a rest period between the operations and DHT administration, because immediately after adrenalectomy or hypophysectomy DHT is poorly tolerated.

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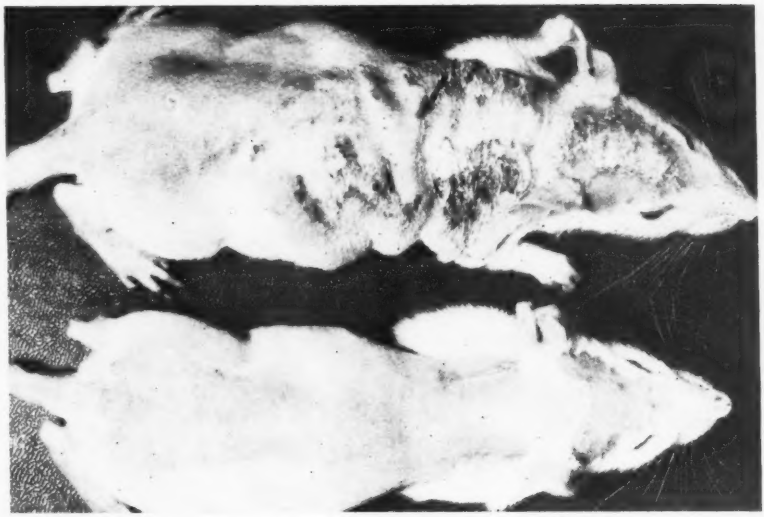

FIG. 1. Intense cutaneous calcinosis at site of plucking (calvarium) and egg-white injection (back) produced by DHT in intact rat (top). These responses are completely inhibited in hypophysectomized animal (bottom). The minor subcutaneous calcification produced by $FeCl_3$ is not visible on skin surface.

Twenty-four hours after DHT administration (that is, during the "critical period") the *hair was plucked* over the calvarium in the animals of all groups; immediately afterwards 2 cc. of a 50% aqueous solution of *egg white* was injected subcutaneously in the lumbar region and 150 μg of $FeCl_3$ in 0.2 cc. of water in the sacral region.

The experiment was terminated on the eighth day by killing all surviving animals with chloroform. The intensity of the cutaneous calcification was estimated in terms of an arbitrary scale: 0 (no lesion), 1 (just detectable lesion), 2 (moderate lesion) and 3 (maximal lesion). The mean readings are given in Table 1 together with standard errors and the percentual mortality rate.

RESULTS

In the rats treated with DHT alone (Group 2), intense calcification was macroscopically visible in the regions challenged by epilation, egg white and $FeCl_3$, as soon as two days after this topical challenge. Both the intact (Group 3) and the adrenalectomized (Group 4) rats treated with DHT and triamcinolone showed even somewhat more pronounced skin lesions. In addition, the adrenalectomized rats became very ill and all but one of them died before the end of the experiment. By contrast, the similarly-treated hypophysectomized rats (Group 5) supported the treatment very well; all of them survived and none exhibited any calciphylactic response at the skin sites challenged by plucking or egg white (Fig. 1). $FeCl_3$, however, did elicit slight calcification even after hypophysectomy.

At this dose level, DHT produced only negligible cardiovascular and renal calcification in a few of the intact and in the one surviving adrenalectomized animal. No such lesions occurred in the hypophysectomized rats. Histochemical investigation of tissue specimens (fixed in alcohol-formol and stained with the v. Kóssa-$AgNO_3$ technique) merely confirmed the macroscopic findings.

An additional experiment was then performed to verify whether hypophysectomy could also protect against a more chronic DHT-intoxication. Ten normal and ten hypophysectomized rats were treated as those of Groups 3 and 5 of the first series, respec-

TABLE 1. PREVENTION OF CALCIPHYLAXIS BY HYPOPHYSECTOMY

Group	Systemic treatment	Topical response to:			Mortality (%)
		Plucking	Egg white	$FeCl_3$	
1	None	0	0	0	0
2	DHT	2.0±0.17	2.2±0.2	1.2±0.20	0
3	DHT+Triamcinolone	2.8±0.17	2.8±0.1	1.1±0.10	0
4	DHT+Triamcinolone +Adrenalectomy	3.0*	3.0*	1.0*	90
5	DHT+Triamcinolone +Hypophysectomy	0	0	0.6±0.12	0

* One survivor only.

tively; but here, DHT was given at the dose of 150 μg in 0.5 cc. of corn oil, daily, from the third to the tenth days after hypophysectomy and the skin was stimulated (plucking, $FeCl_3$ and egg-white injection) on the tenth day. Since this is not the "critical period," calciphylaxis was mild even in the controls. Still, autopsy on the 15th day again revealed complete suppression by hypophysectomy of whatever cutaneous calcinosis was otherwise induced by plucking and egg white, but only partial inhibition of that caused by $FeCl_3$. The calcification of the heart and renal cortex, normally elicited by DHT under these conditions, was likewise suppressed by hypophysectomy; yet curiously, calcification in the aorta and renal papilla was actually aggravated.

DISCUSSION

That triamcinolone fails to inhibit the calciphylactic response is merely an additional indication of the essential difference between this form of reaction and inflammation. It is especially noteworthy, however, that hypophysectomy offers such striking protection against certain forms of calciphylaxis. Necrotic tissue notoriously exhibit a tendency to calcify, especially when the blood calcium is high; but calciphylaxis is apparently a much more active phenomenon that does not depend merely upon physicochemical factors governing the calcium avidity of necrotic tissues. This view is supported by the facts that: (1) The challenging agents themselves cause no necrosis. (2) The attraction of calcium goes far beyond the mere saturation of the challenged area; it spreads and causes considerable thickening of the skin accompanied by edema and secondary necrosis. (3) The development of calciphylaxis depends upon hormonal stimuli of hypophyseal origin.

Further experiments are now under way to identify the hypophyseal factor that makes this reaction possible.

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ERRATA

In the article by Villee, Engel, Loring and Villee, *Endocrinology* **69**: 354–372, 1961, the name of the compound appearing on page 371, line 10 as 3β, 16α-dihydroxy-5α-pregnane-20-one should read 3α, 16α-dihydroxy-5α-pregnane-20-one.

In the article by Ball and Merrill, *Endocrinology* **69**: 596–607, 1961, the legends to Figs. 1 and 3 should read 0.15 units in place of 0.15μ units.

THE ENDOCRINE SOCIETY

1962 Annual Meeting

The Forty-Fourth Annual Meeting of The Endocrine Society will be held in the Palmer House, Chicago, Illinois, Thursday, Friday and Saturday, June 21-22-23, 1962.

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily, and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 22 at 7:30 P.M. preceded by cocktails at 6:30 P.M.

The Chairman of Local Arrangements is Dr. Allan T. Kenyon.

All members are urged to make their hotel reservations immediately. The Palmer House will hold 300 bedrooms for members until May 1, 1962, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations early, directly with the hotel advising them of time and date of arrival and departure. If you plan on remaining for the A.M.A. Meeting, please advise the hotel. Make your reservations early and avoid disappointment. Hotel reservation card will be mailed to members shortly after January 1, 1962.

Everyone attending the meetings is required to register. By action of the Council, non-members will be charged a registration fee of $15.00 for any part of the scientific sessions. Members will be charged a registration fee of $5.00.

Graduate students, residents and interns and members of the medical departments of the armed forces will be admitted upon presentation of proper credentials CERTIFIED BY DEPARTMENT HEAD OR SUPERINTENDENT OF HOSPITAL and payment of $5.00 registration fee.

Final program, membership card and advance registration forms will be sent on May 15, 1962 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send an original and four copies of the title and abstract to

the Vice-President, Dr. Leslie L. Bennett, Dept. of Physiology, University of California Medical Center, San Francisco 22, California, not later than February 15, 1962. It is imperative that the abstracts be informative and complete with results and conclusions—not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately two hundred abstracts submitted each year and compiling of the program from these involves a tremendous amount of time and effort on the part of the Program Committee.

THE COUNCIL REQUESTS THAT AUTHORS ADHERE STRICTLY TO THE FOLLOWING RULES WHEN SUBMITTING ABSTRACTS, OTHERWISE THEY CANNOT BE CONSIDERED:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.
2. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgements to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas, graphs or extensive tables cannot be used.
3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, e.g., M.D., of each author should be written after his name.
   Line 3. Institution of origin and city in which institution is located.
4. Authors who wish receipt of their abstracts acknowledged should enclose self-addressed post card with the title of the abstract noted thereon.
5. Authors are also to enclose 3×5 filing cards, one for each author, giving his name and initials in the upper right hand corner, and the complete title of the abstract with all the authors listed in the center of the card. There should be a second card enclosed listing the title and all authors and mailing address of the senior author. This is necessary for indexing. Senior authors will be notified of the acceptance of their papers for the final program.

6. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. The original and four copies should be forwarded to Dr. Bennett.
7. Abstracts should be letter perfect, since there will be no opportunity for proof reading by the authors.

1962 AWARDS

Fred Conrad Koch Award

In 1957 a substantial legacy was bequeathed to the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957, Dr. I. L. Chaikoff in 1958, Dr. Wilbur W. Swingle in 1959, Dr. Emil Witschi in 1960 and Dr. Lawson Wilkins in 1961. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator who has not attained his 36th birthday by June 1st of the year the award is presented, in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. Jane A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951

Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer, 1959 Dr. Gordon L. Farrell, 1960 Dr. Don H. Nelson and 1961 Dr. Ernst Knobil. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. Each Fellowship is awarded on alternate years and the stipend, which will not exceed $7,500 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Upjohn Awards

The Council of The Endocrine Society has approved a change in status of the Upjohn Scholar to that of The Upjohn Awards.

There will be two awards in the amount of $1,250 each, one for the best article in format and content printed in ENDOCRINOLOGY and one in THE JOURNAL OF CLINICAL ENDOCRINOLOGY AND METABOLISM during the preceding year.

Awardees will be chosen by the Awards Committee at its regular meeting in November of each year.

NOMINATIONS

Nominations for the Fred Conrad Koch Award, the Ciba Award, and the Ayerst and Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than October 15 each year.

ENDOCRINOLOGY STUDY SECTION

The Endocrinology Study Section, Division of Research Grants, National Institutes of Health, has the following purified radioactive steroids available for distribution to qualified investigators, free of charge except for $5.00 per ampule to cover handling and shipping. The materials are stored at Tracerlab, Inc., Waltham, Massachusetts, and the recipient will be billed by Tracerlab at the rate of $5.00 per ampule.

Material	*Batch*	*Specific Activity*
Hydrocortisone 4-C^{14}	ES-F-10-1	8.7 μc/mg
Hydrocortisone 4-C^{14}	ES-F-50-1	42.4 μc/mg
Corticosterone 4-C^{14}	ES-B-25-1	22.8 μc/mg
Corticosterone 4-C^{14}	ES-B-50-1	44.4 μc/mg
7-^{3}H Aldosterone		20. μc/μg
7-^{3}H Cortisol		28. μc/μg

Investigators may obtain supplies of these materials by sending a brief description of the proposed usage, together with an estimate of the amount of materials required. Requests are filled as promptly as possible after a review by the Committee on Radioactive Hormone Distribution.

The 4-C^{14} compounds are sealed in ampules containing 5 μc, 10 μc, or 25 μc each, in a solution of 95% benzene and 5% methanol, and are about 96% pure. The 7-^{3}H steroids are also in a solution of benzene and methanol, the Aldosterone sealed in 60-μc ampules, and the Cortisol in 30-μc ampules. Investigators are cautioned to calibrate each batch of material before use.

The applicant's Atomic Energy Commission registration number governing the use of isotopic materials should accompany all requests. In addition, a voucher number or purchase order certifying that the handling charges will be paid to Tracerlab, Inc., together with the exact address of the applicant, would be appreciated. This will avoid loss or undue delay, and will assist Tracerlab in collecting the handling charge due them for processing materials.

Requests for these compounds should be submitted to:

Morris M. Graff, Executive Secretary
Endocrinology Study Section
Division of Research Grants
National Institutes of Health
Bethesda 14, Maryland

The Endocrinology Study Section, Division of Research Grants, National Institutes of Health, has the following highly purified pituitary hormones available for distribution free to qualified investigators:

Growth Hormone, bovine, non-sterile, for animal experiments only. Bulk; dispensed as requested.

Follicle-stimulating hormone, ovine, sterile preparation. 25 mg. vials for experiment; 5 mg. vials for assay standard.
Prolactin, ovine, sterile preparation. 25 mg. vials.

Investigators may obtain supplies of these hormones by sending a brief description of the proposed usage, together with an estimate of the amount of materials required. Requests are filled as promptly as possible from supplies maintained by Dr. A. E. Wilhelmi of Emory University, Atlanta, Georgia, after review by the Committee on Hormone Distribution. These hormones are prepared and distributed under a program generously supported by the National Institutes of Health.

Requests for these materials should be submitted to:

Morris M. Graff, Executive Secretary
Endocrinology Study Section
Division of Research Grants
National Institutes of Health
Bethesda 14, Maryland

The Van Meter Prize Award for 1962

The American Thyroid Association, Inc. again offers the Van Meter Prize Award of $500.00 to the essayist submitting the best manuscript of original and unpublished work concerning "Goiter—especially its basic cause." The studies so submitted may relate to any aspect of the thyroid gland in all of its functions in health and disease. The Award will be made at the Annual Meeting of the Association at the Roosevelt Hotel, New Orleans, Louisiana, May 9–12, 1962. A place on the program will be reserved for the winning essayist if he can attend the meeting. When more than one author's name appears on the manuscript the authors will be asked to designate a single recipient to receive the award.

The competing essays may cover either clinical or research investigations, should not exceed 3000 words in length and must be presented in English. Duplicate, typewritten copies, double spaced, should be sent to the Secretary; Theodore Winship, M.D., 430 N. Michigan Ave., Chicago 11, Illinois, *not later than January 1, 1962*. The committee who will review the manuscripts is composed of men well qualified to judge the merits of the competing essays.

THE WORLD MEDICAL ASSOCIATION

The United States Committee of The World Medical Association is sponsoring a project to send used medical journals to physicians in Asia. Specialty journals are needed, particularly this one.

If you wish to participate in this doctor-to-doctor program, please write to The World Medical Association, United States Committee, 10 Columbus Circle, New York 19, N. Y., listing the journal(s) you wish to contribute.